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A study of triglyceride metabolism in Mycobacterium smegmatis.

Chun-Kwan Wun
University of Massachusetts Amherst

A STUDY OF TRIGLYCERIDE METABOLISM IN *MYCOBACTERIUM SMEGMATIS*

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by

CHUN-KWUN WUN

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by

CHUN-KWUN WUN

Approved as to style and content by:

Robert W. Walker
(Chairman of Committee)

J. A. Smith
(Head of Department)

C. M. Gawinowski
(Member)

W. H. [illegible]
(Member)

September 1974

Affectionately dedicated to

my parents and my wife

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A Study of Triglyceride Metabolism in
Mycobacterium smegmatis (September 1974)

Chun-Kwun Wun, B.S., Chung Chi College, Chinese
University of Hong Kong
M.S., Springfield College
M.S., University of Massachusetts
Directed by: Dr. Robert W. Walker

In mammalian tissues three pathways have been delineated as being involved in triglyceride (TG) biosynthesis: 1. the sn-glycerol 3-phosphate (α-GP) pathway; 2. the monoglyceride (MG) pathway; 3. the dihydroxyacetone phosphate (DHAP) pathway. Previous work from our laboratory has shown that the MG pathway does not exist in *M. smegmatis*. The relative importance of the other two pathways in TG formation was investigated in this study.

Experiments performed in this study with labeled glucose, fructose 1,6-diphosphate and 3-phosphoglycerate have shown that the above compounds and in particular the glycolytic intermediate DHAP were not acylated to any extent. On the other hand, indirect as well as direct evidence was obtained indicating that α-GP acts as a primary acyl group acceptor in TG synthesis.

α-GP-acyltransferase activities were found in both the pellet and supernatant fractions of *M. smegmatis*

cell-free extracts. Their activities were found to be enhanced by low concentrations of ATP, CTP, and Mg^{2+}. Inhibition of this group of enzymes was also observed with flouride ion (F^-), Tween-20, and after freezing and thawing.

The enzyme phosphatidate (PA) phospholydrolase which is two separate enzymes in animal systems, appears to be a single entity in the bacterial system. This enzyme was found to be totally Mg^{2+}-dependent, stimulated by Na^+ and K^+ and inhibited by Tween-20 and F^-.

An endogenous TG precursor was detected in the cell-free extracts of M. smegmatis. Indirect and direct evidence was presented showing that such a compound was a 1,2-diglyceride (DG). The DG-acyltransferase activity was found to be much greater in the supernatant (cytosol) fraction than in the pellet (membrane) fraction of the cell-free extracts. Its activity was found to be stimulated by ATP, CTP, Mg^{2+}, and Na^+, and was inhibited by repeated freezing and thawing.

Enhancement of TG synthesis was observed with various glycolytic products. Experimental results suggest that the stimulatory action of these compounds was of an indirect nature, being a result of the stimulatory effect of the ATP generated as a result of the metabolism of these glycolytic compounds.

A possible method of purification or partial purification of the important enzyme, PA-phosphohydrolase was suggested.

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INTRODUCTION

The genus Mycobacterium is comprised of both saprophytic and pathogenic bacteria. The pathogenic members of this genus are the causative agents of two devastating human diseases, tuberculosis and leprosy. Early in the studies of these organisms, a close correlation was observed between the high lipid content of the organisms and pathogenesis.

In addition to having a high lipid content (ranging up to 30% of the cell dry weight in some species) these organisms also produce a great variety of lipid compounds (9, 44, 99). Many of these compounds are unique to the mycobacteria.

The mycobacteria also appear to be the only group of bacteria* which produce triglycerides. The cells may contain a very high concentration of this lipid, in some instances it constitutes the bulk of the lipids.

With the exception of a study of the positional distribution of fatty acids on mycobacterial triglycerides (157), the triglycerides have remained the least studied of

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*Although most species of mycobacteria are bacterium-like in appearance, these organisms are classified with the fungus-like organisms the nocardia, actinomycetes, and streptomycetes (15).

the mycobacterial lipids. The pathways involved in triglyceride biosynthesis and factors regulating these pathways are not known. This study was instituted to provide information on the biosynthesis of triglycerides by the mycobacteria.

LITERATURE REVIEW

The triglycerides (TG) comprise a major portion of the lipids of the mycobacteria. Although quantitative data is lacking for most strains (9) the TG content of the few strains studied has been found to comprise up to 30% of the cell dry weight (44). In unpublished studies (11) it was shown that when Mycobacterium smegmatis was grown on a medium containing high concentrations of glycerol, TG was found to constitute 13% of the cell dry weight. At lesser glycerol concentrations, the TG content was somewhat reduced. However, even when the cells were cultivated with asparagine and citrate as sole carbon sources, the cells produced a few percent of TG.

Mycobacterial TGs have been found to contain a high proportion of long-chain fatty acids (over C_{20}). A stero-specific analysis of the fatty acid distribution of triglycerides isolated from M. bovis BCG and M. smegmatis (157) showed that the fatty acids of position 1 were principally oleic; position 2 palmitic; and position 3 tetracosanoic in the case of M. smegmatis and hexacosanoic in the case of M. bovis BCG. The fatty acid distribution of the phospholipids (phosphatidyl ethanolamine and cardiolipin) was essentially the same as that

of the TGs. Most of the oleate was replaced by tuberculosterate (10-methylstearic acid, synthesized by the C-methylation of oleate) and much of the palmitate at position 2 of the phospholipids was found to have been desaturated to the 16 carbon monoene (probably the Δ^9 and Δ^{10} hexadecenoates) (67).

The function of TGs in the mycobacteria is not known. They may very likely have a dual function, serving both as a source of energy and carbon and as a source of fatty acids for phospholipid and mycolic acid biosynthesis (157). Johnston and Paultauf (71) and Kornberg (89) suggested that in mamalian systems TGs serve as a fatty acid pool supplying substrate for phospholipid biosynthesis. The TG may also serve as a source of 1,2-diacyl-sn-glycerols (1,2-diglycerides, DG) for the reaction CDP-base + DG → phospholipid-base, the predominant route of phospholipid biosynthesis in mammalian systems (112). If the TGs are involved in the biosynthesis of phospholipids as suggested by these authors, then they may be more intimately involved in overall lipid metabolism than had previously been thought.

It has been well established that there are two major pathways for TG synthesis in animal tissues: 1. the sn-glycerol 3-phosphate (α-GP) pathway which involves α-glycerophosphate as precursor, with phosphatidic acid (PA) and 1,2-diacyl-sn-glycerol as intermediates (Fig. 1) and 2. the monoglyceride (MG) pathway, in which

monoglycerides are directly acylated to di- and tri-glycerides. The first pathway was suggested by Kennedy in 1957 (81) and has since been shown to exist in several different mammalian tissues (19, 85, 86, 139, 142, 143). Evidence for the second pathway was first presented by Clark and Hubscher (24). Their results have been confirmed and extended by various investigators (4, 19, 20, 21, 25, 31, 61, 63, 79, 84, 106, 134, 135, 138, 139, 143, 144, 146).

Recently, another pathway has been found to exist. Dihydroxyacetone phosphate (DHAP) was shown to be acylated to acyl dihydroxyacetone phosphate. This compound was in turn reduced to form lysophosphatidic acid (46, 47, 48, 49). Acylation of lysophosphatidate to phosphatidate has also been demonstrated (98, 147). These reactions provide an alternate pathway for the biosynthesis of PA.

Investigations by Goldman (43) on the pathways of glycerol metabolism in M. tuberculosis $H37R_A$ suggest the possibility of the involvement of DHAP in mycobacterial lipid metabolism. In this study Goldman showed that glycerol was initially oxidized to dihydroxyacetone (DHA) prior to phosphorylation to DHAP and subsequent metabolism.

Figure 1 is a composite chart of the relationships between carbohydrate and lipid metabolism in mammalian and bacterial systems. As can be seen from this figure, PA is the key compound in the biosynthesis of both neutral and

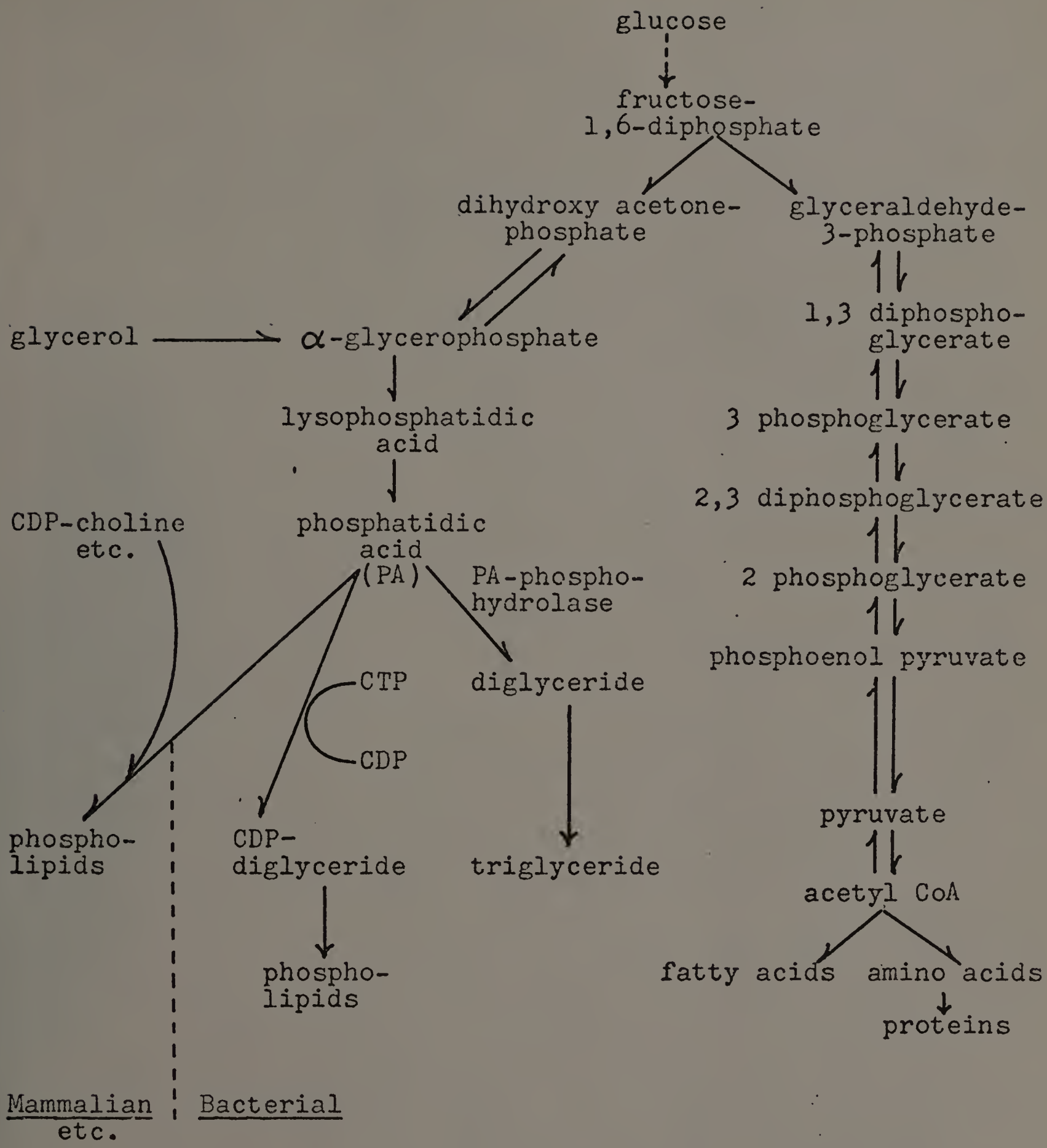

Figure 1

Some Interrelations Between Carbohydrate Metabolism and Lipid Biosynthesis

phospholipids. In mammalian systems the PA is hydrolyzed to DG which either reacts with a CDP-base to form a phospholipid or is acylated to form TG (112). In bacterial systems the PA is converted to CDP-DG which is used in phospholipid biosynthesis (23).

The pathways involved in neutral and phospholipid biosynthesis in the mycobacteria are not known. Unpublished observations (Walker, 1972) have shown, however, that a membrane preparation of M. smegmatis was able to form CDP-DG from PA and CTP suggesting the involvement of the typical bacterial pathway in phospholipid biosynthesis. Other observations (11) have shown the presence of the sn-glycerol-3-phosphate pathway and the absence of the MG pathway.

I. Acylcoenzyme A: sn-glycerol 3-phosphate-o-transferases

1. Distribution of the Acyltransferases. The properties and distribution of acylCoA: sn-glycerol 3-phosphate-o-transferases have been studied in various mammalian tissues. Their activities were detected in adipose tissue (8, 30, 68, 133, 158, 159), liver (29, 39, 88, 93, 97, 98, 110, 122, 159, 164), intestinal mucosa (19), mammary gland (90), and brain cerebral hemispheres (123). Their kinetic parameters were also investigated in bacterial cell-free preparations (3, 41, 117, 130), and in yeast (71).

Although it is generally accepted that the activities of the acyltransferases are present in various subcellular fractions, their quantitative distribution pattern is controversial; Daniel and Rubinstein (30), and Roncori and co-workers (8, 133) found these enzymes were largely confined to the mitochondria, Steinberg (145), Tzur and Shapiro (151), Lamb and Fallon (93), Yamashita and Numa (164), Eibl et. al. (34), and Possmayer et. al. (122) reported that the enzymes were primarily distributed in the microsomal fraction. In a study of lipid metabolism in the yeast Saccharomyces carlsbergensis Johnston and Paltauf (71) concluded that the sn-glycerol 3-phosphate acyltransferase activities were equally distributed between the mitochondria and the mitochondria-free supernatant fractions.

Working with rat liver, Daae (29) provided evidence that the activities of sn-glycerol 3-phosphate acyltransferases were present in both microsomes and mitochondrial outer membranes, and the main product of the esterification reaction was phosphatidic acid and 1-acyl-sn-glycerol 3-phosphate, respectively. He suggested that the failure of other workers to find appreciable acyltransferase activity in the mitochondrial system may have been due to the extreme sensitivity of these enzymes to inhibition and to the fact that the main product was lysophophatidate

which may have escaped detection.

2. Are Singular or Multiple Enzymes Responsible for the Acylation of the 1 and 2 positions of sn-glycerol 3-phosphate? Ray et. al. (130) reported that a single multifunctional enzyme was responsible for the acylation of both the 1 and 2 positions of the sn-glycerol 3-phosphate molecule in PA-biosynthesis. This view was supported by the fact that phosphatidic acid was identified as the sole product of the esterification reaction (68, 88, 122).

With the exception of a few bacterial systems (3, 41) and the rat liver microsome system of Lamb and Fallon (93) attempts to demonstrate the presence of the intermediate lysophosphatidic acid, have been unsuccessful (38, 53, 54, 80, 97, 103, 122, 147)

In 1963, Merkl and Lands (107) suggested that a number of acyltransferases may be involved in lipid synthesis. Later, widely differing patterns of acyltransferase activities, both with different acylcoenzyme A and with different phospholipids, were found with microsomal preparations from various tissues by Lands and Hart (98). They also reported that the enzyme which catalyzed the first acylation of glycerol phosphate differed from the enzyme catalyzed the second acylation by being more sensitive to inhibition by sulfohydryl-binding reagents. It was proposed that the process of PA formation was mediated by two distinct

acyltransferases and occurred by a sequential acylation of glycerol phosphate.

Recently the monoacylation of sn-glycerol-3-phosphate has been demonstrated in a number of studies (29, 39, 110, 117, 153), evidence was presented that lysophosphatidate does indeed serve as an intermediate in the formation of PA.

Yamashita and Numa (164), by resolving the rat liver microsomes with a non-ionic detergent Triton X-100, in glycine buffer pH 8.6, and by using the separation procedures molecular-sieve chromatography and sucrose density gradient ultra-centrifugation, were able to partially purify a glycerol phosphate acyltransferase which catalyzed the formation of 1-acyl-glycerol-3-phosphate, further substantiating that more than one acyltransferase is involved in the process of PA biosynthesis from acyl derivatives and glycerol phosphate.

3. Random or Stereospecific Acylation of sn-glycerol Phosphate. Naturally occurring lipids seldom have a random fatty acid distribution (51). This fact is particularly evident by the work of Rhodes (131) on egg yolk phospholipids. Walker, Barakat and Hung (157) while studying the positional distribution of fatty acids in the phospholipids and triglycerides of mycobacteria, observed that position 1 of these lipid fractions was esterified principally with C_{18} related fatty acids while position 2 was

occupied mainly by C_{16} fatty acids. A preponderance of very long chain fatty acids were found on the 3-position of the triglyceride molecules.

Lands (94), in 1958, had suggested that the acylation of glycerol phosphate was random and the specific fatty acid distribution pattern observed in tissue phospholipids might arise from a redistribution of acids after nitrogenous base has been attached to the molecules. The presence of and the properties of different acylCoA : phospholipid acyltransferases have been described (95, 98, 107). It is possible that the fatty acid pattern observed in the natural triglycerides is not introduced at the initial acylation steps but is a result of the action of the lipases and the re-acylation reaction.

Pieringer *et. al.* (117) studied the acylation of sn-glycerol-3-phosphate by *E. coli* particulate fractions using various acylCoA derivatives. They reported that certain fatty acids which are reportedly not constituents of the glycerolipids in this organism were used as active substrates in the esterification reaction. These observations would cast doubts on the selectivity of the glycerol-phosphate acyltransferases.

Questions of positional specificity of acylCoA-glycerol phosphate acyltransferases were raised by Lands and Hart (97, 98) and Hill *et. al.* (54). They reported that

although non-random fatty acid incorporation was observed when a mixture of linoleyl- and stearoyl-CoA and sn-glycerol-3-phosphate was incubated with rat liver slices, four species of PA were produced when the same mixture was incubated with a solubilized pigeon liver microsome preparation. The finding that 21% of 1-stearoyl-2-linoleyl- and 22% of 1-linoleyl-2-stearoyl-glycerol-3-phosphate and 15% and 42% of distearoyl and dilinoleyl species, respectively, were formed, indicated that the cell-free system was not selective in esterifying these two acids, and that both positions 1 and 2 may be acylated by either acid.

Studies showing that there was no preference for unsaturated fatty acylCoA in the acylation of 1-monoacylglycerol-3-phosphate (12, 41), or for saturated acyl donors in the acylation of the 2-acyl isomer (114) have been reported. Such an apparent lack of selectivity, however, might be due to the elevated concentration of substrates used in <u>in vitro</u> experiments was suggested by Lands and Okuyama (96). These authors demonstrated that saturated fatty acids could be progressively excluded from the 2-position of phosphatidate molecules in the presence of unsaturated acylCoA by decreasing the concentration of 1-acylglycerol-3-phosphate.

Substrate specificity in the esterification of sn-glycerol-3-phosphate in cell-free systems was first described

by Kornberg and Pricer in 1953 (88). In 1966, positional specificity of various fatty acids in the acylation reaction was observed by Husbands and Reiser (66). Possmayer and co-workers (122), using rat liver microsomes and ATP, coenzyme A and ^{14}C-fatty acids as the acyl donor system, found palmitate to be the most rapidly incorporated saturated acid, but an even faster rate of acylation was obtained when unsaturated acids of 18 carbon atoms were used. When the distribution of the radioactivity incorporated into phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid was studied, it was shown that the majority of the saturated fatty acids were at the 1-position, while the polyunsaturated fatty acids were essentially confined to the 2-position. Similar results were obtained with rat liver microsomes by Lamb and Fallon (93) and Monroy *et. al.* (110), and with *E. coli* particulate preparation by Ray and co-workers (130) and Van Den Bosch and Vagelos (153), and with partially purified rat liver microsomal enzymes by Yamashita and Numa (164).

Supporting data from *in vivo* studies were provided by Akesson, Elovson, and Arvidson. Radioactive linoleate (5), glycerol (6), or palmitate (7) was injected into rats by the intraportal route and the rates of incorporation into various hepatic lipid fractions was measured. It was reported that 90% or more of ^{14}C-linoleate and of ^{3}H-palmitate

was incorporated into position 2 and position 1, respectively. When the radioactive unsaturated fatty acid was used, labeling was much greater in palmitate- than in stearate- containing species. When ^{3}H-unsaturated fatty acid was used, the highest labeling was found in saturated:monoenoic and saturated:dienoic species. The incorporation of ^{3}H-glycerol was shown to occur predominantly in the species containing palmitate on the 1-position and either a monoenoic or dienoic acyl group on the 2-position.

4. <u>The Possible Regulatory Role of AcylCoA:sn-glycerol-phosphate-o-transferases in the Biosynthesis of Lipids</u>. Lipids make up a significant portion of biological membranes. Since these dynamic membrane systems are continuously undergoing metabolic flux, it is apparent that the formation and degradation of the lipid components must be under some form of tight control. The bulky, hydrophobic nature of the lipid molecules makes it unlikely that they could function in feedback control. In addition, the change in enzyme profile of the cell would seem to be too clumsy a process to respond to the ever changing nature of the membranes. It is likely that certain small, hydrophilic molecules are responsible for modulating the rate at which these hydropholic moieties are synthesized.

The esterification of sn-glycerol-3-phosphate by long chain fatty acids has been suggested as a possible

regulatory step in lipogenesis (16, 38, 60, 152). Effects of hydrophilic molecules on enzymes catalyzing such a reaction have also been reported by various investigators. Pieringer and associates (117) observed that the process of acylating glycerol phosphate to lysophosphatidate and then to phosphatidate was dependent on the presence of Mg^{2+}. Similar results were also reported by Fallon and Lamb (39), by Jamdar and Fallon (68), and by Yamashita and Numa (164). Although it is generally agreed that magnesium is a positive effector, contrasting results have been obtained when some other divalent ions were tested. Jamdar and Fallon (68) showed that acyltransferase activity was reduced 25-40% by Ca^{2+}, Fe^{2+}, Co^{2+}, and Sn^{2+}, while Zn^{2+} and Cu^{2+} caused a 90% decrease in activity. On the other hand, Yamashita et. al. (164) reported that the partially purified glycerolphosphate acyltransferase required Ca^{2+} for its activity, and Mn^{2+} and Co^{2+} were able to substitute for Ca^{2+} with varying degrees of effectiveness.

Data indicating that acyltransferase activity was stimulated by albumin at low concentration of acyl coenzyme A was presented by Fallon and Lamb (39).

The ability of cytidine triphosphate to decrease the incorporation of ^{32}p-glycerol 3-phosphate into rat brain lipids was first reported by McMurray et. al. (113). This

observation was extended to other cytidine nucleotides by Possmayer and Strickland (124, 125, 126). Although the action of cytidine nucleotides was suggested to be by inhibition of the glycerol phosphate acyltransferase enzymes, the stimulation of the phosphatidate phosphohydrolase enzyme was not ruled out by these authors.

It is possible that cytidine nucleotides stimulate the formation of CDP-diglycerides from PA, and the CDP-DG is hydrolyzed to produce CDP and DG. In fact, evidence for such a hydrolysis has been presented by Kiyasu (87), and results showing that CTP and CMP stimulate neutral lipid formation at the expense of PA have been obtained by Erbland et. al. (37) and by Marinetti and co-workers (32, 33, 102).

Recently Possmayer and Mudd (123) determined that the incorporation of sn- ^{14}C-glycerol 3-phosphate into rat brain cerebral hemispheres total lipid was markedly decreased by cytidine nucleotides, particularly CTP. It was shown that the depression was due to a striking decrease in the labeling of PA. Evidence was also provided that the phenomenon was not mediated by a stimulation of PA-phosphohydrolase or by the hydrolysis of CDP-DG. Thus, the inhibitory effect of cytidine nucleotides on the glycerol phosphate acyltransferases was evident.

Merlie and Pizer (108) in a study of phospholipid

formation in E. coli, found a strict correlation between guanosine tetraphosphate accumulation and inhibition of the biosynthesis of these lipids. Experimental data was presented which suggested that the regulation occurred at the sn-glycerol-3-phosphate acyltransferase step.

In addition to the pyridine- and purine-nucleotides, a number of other hydrophilic effectors of the acyltransferases have been identified. Sulfhydryl binding agents (39, 98), and sodium palmitate (39) have also been shown to have marked effects on the enzymes.

The activity of the acyltransferases has also been found to be affected by lipid materials. PA (39), phosphatidyl serine, and phosphatidyl inositol (164) were reported to be inhibitory to the enzymes, while stimulation of their activity by phosphatidyl choline and phosphatidyl ethanolamine was demonstrated (164). Working with microsomal and whole cell preparations obtained from adipose and intestinal tissues, Polhein et. al. (121) reported that the 2-monoleyl ether and various monoglycerides were inhibitors of PA and neutral glycerides synthesis. These authors determined that the inhibitory effect of these molecules was due to their inhibition of the acylation of glycerol-phosphate.

II. Dihydroxyacetone Phosphate Pathway

1. General Consideration. The dihydroxyacetone phosphate pathway was first described by Hajra and associates. In a study of the rapid labeling of mitochondrial lipids by $^{32}P_i$ or γ-^{32}P-ATP, Hajra, Sequin and Agranoff (50) noted that a lipid was formed which had properties not corresponding with those of any known phospholipids. This unknown mitochondrial lipid was subsequently identified as acyldihydroxyacetone phosphate (48). Its formation from DHAP and acyl coenzyme A in guinea pig liver, brain, kidney, and heart (47) and in rat liver, brain, kidney, heart, testis, spleen, and adipose tissue (92) has been reported. Evidence that this lipid was reduced to lysophosphatidate by NADPH in liver mitochondria and in ascites tumor cell microsomes was provided by Hajra and Agranoff (49), and LaBelle and Hajra (91), respectively. The formation of phosphatidic acid and neutral lipids from DHAP, acyl CoA, and pyridine nucleotides has been demonstrated in a number of recent studies (91, 127, 129).

The existence of the DHAP-pathway as an alternate route for phosphatidate-biosynthesis was further documented by Rao, Sorrels, and Reiser (128, 129). They found that the formation of PA and glycerolipids from ^{14}C- fructose 1,6-diphosphate was stimulated by the mitochondria-free supernatant fraction by a process that required NADH or

NADPH. This reaction was not affected by dilution upon addition of glycerol 3-phosphate. They also reported that PA-synthesis from DHAP in rat liver was influenced by fasting and re-feeding, while PA-synthesis from glycerol phosphate was not responsive to changes in diet.

The relative participation of the two pathways in mouse liver homogenate has been assessed by Agranoff and Hajra (2). Based on the reported nucleotide specificity for the reduction of DHAP and of acyl-DHAP, ^{3}H-NADH and ^{3}H-NADPH were used in the estimation of the relative rate of the two pathways. Results obtained indicated that a significant portion of glycerolipids could arise via the DHAP-pathway. However, the validity of such a conclusion was questioned by McMurray and Magee (112). In fact, using a mixture of 2-^{3}H- and 1,3-^{14}C- glycerols and by measuring the ratio of ^{3}H:^{14}C, Okuyama and Lands (115) have established that the biosynthesis of PA in rat liver slices cannot proceed via the DHAP-pathway. Thus, the physiological significance of DHAP as a direct substrate for acylation awaits further investigation.

2. Enzymes of the DHAP-pathway. The activity of acylCoA-DHAP acyltransferase was found to be higher in mitochondrial fractions than in microsomal fractions of liver tissues from both guinea pigs (47) and rats (92). The reduction of acyl DHAP to 1-monoacylglycerol 3-phosphate

has been reported to take place in mitochondria (49), and in both mitochondria and microsomes (91). Enzymes catalyzing this step were found to be specific for NADPH as the coenzyme (49, 91). However, Puleo, Rao, and Reiser (127) provided some evidence that enzymes of the DHAP-pathway leading to the formation of PA in rat liver were present in the microsomal fraction but were absent from mitochondria. Furthermore, NADH was reported to be twice as active as NADPH as a co-factor in this biosynthetic reaction. Recently, the formation of glycerolipids from DHAP, acylCoA, NADPH or NADH in rat liver mitochondria and microsomes was investigated by LaBelle and Hajra (92). It was suggested that the major portion of the glycerides formed in the presence of NADH was a result of the acylation of α-GP which had been formed from DHAP by action of α-GP dehydrogenase.

3. Stereospecific Distribution of Fatty Acids in Respect to the DHAP-pathway. In a study which analyzed the fatty acids distribution patterns of phosphatidic acids, Hajra (46) reported that PA derived from DHAP showed a pattern similar to that of natural glycerides and phosphoglycerides, while that formed from α-GP was more random. Although conclusive evidence was not obtained as to whether the DHAP-pathway accounts for the natural positional specificity, greater selectivity appear to be found via the

DHAP-pathway than via the α-GP-pathway.

III. Phosphatidate Phosphohydrolase

1. General Consideration. The enzyme phosphatidate phosphohydrolase which was first discovered in plant tissue by Kates (77) in 1955 was subsequently identified in animal tissues by Smith, Weiss, and Kennedy (141, 160). This enzyme, in addition to being a required enzyme for the continuity between phosphatidic acid and 1,2-diglyceride in the α-GP-pathway of triglyceride snythesis, has also been implicated in the active transport of sodium and potassium (25, 27, 36, 56, 57, 58). Evidence has also been presented that PA-phosphohydrolase is involved in a regulatory role in triglyceride and phospholipid metabolism (111, 156).

It appears that there are at least two types of PA-phosphohydrolases, namely, the particulate or tightly membrane-bound enzyme, and the soluble or loosely membrane-bound enzyme. The existence and properties of the particulate PA-phosphohydrolase in the microsomal, mitochondrial and liposomal fractions of various animal tissues have been studied extensively. The activity of this enzyme has been detected in mammalian liver (120, 136, 137, 163), brain tissues (1, 59, 111, 148), kidney (27, 28), intestinal mucosa (19, 27, 62, 74), and adipose tissue (68, 69, 146). Its

occurrence in erythrocyte membrane (57, 58) and avian salt gland (56) was also reported.

2. Nature and Properties of the Enzyme. The lipoprotein nature of this enzyme was suggested by the findings of Agranoff (1) and Coleman and Hubscher (27). They found that the enzyme activity was greatly diminished by treatment with n-octanol or n-butanol. This deactivation could be overcome by the addition of a lipid preparation (28). Coleman et. al. (27) observed that the enzyme was inhibited by *p*-chloromercuribenzoate, and inhibition was reversed by excess glutathione indicating that the -SH groups are essential for its activity.

The substrate specificity of this enzyme was also studied by these workers. Based on the observations that sn-glycerol-3-phosphate and glycerophosphatide with monoesters of phosphoric acid linked to inositol were not hydrolyzed, these investigators concluded that the free hydroxyl groups of glycerol had to be esterified before the phosphatidic acid ester bond could be cleaved by this enzyme. Furthermore, the monoester bond of phosphoric acid must be near the diglyceride moiety of the molecule and probably directly esterified with it if hydrolysis was to take place. It was also reported that when the phosphoric acid was further substituted, negligible hydrolysis of the bond was observed. Similar observations have been made by Johnston

et. al. (74) and by Agranoff (1). It was also suggested that the chain length and degree of saturation of the substituting fatty acids might influence the rate of the reaction. Whether this is due to the solubility of the substrate or due to the enzyme specificity was not explained.

The optimal pH reported was generally in the range of 6 and 7 (1, 27, 136). Increased enzyme activity at pH 8.1, however, has been observed (1). This increase in activity, however, was thought to be due to the increased solubility of the substrate.

The effects of certain detergents on the enzyme activity have been studied by various workers. Coleman and Hubscher (27) found that strong inhibition was exerted by sodium dodecylsulfate, Cetarlon, Teepol, Tween-20, deoxycholate and cholate. Similar results were reported by Sedgwick and Hubscher (136). However, the latter authors observed that although the nonionic detergent Tween-20 inhibited the enzyme slightly at low concentration, it stimulated the activity considerably when high concentration was employed. A strong inhibitory effect of high concentration of Tween-20, on the other hand, was shown by Brindley and Hubscher (19).

The reported effects of monovalent, divalent metal ions as well as that of fluoride ion on the enzyme activity were

observed to be quite variable. Agranoff (1) working with phosphatidate phosphohydrolase of brain homogenate, showed that NaF, reportedly a strong inhibitor (27, 58, 137), was ineffective against this enzyme. KCl and NaCl were also found to have no effect in altering the enzyme activity. Others, however, have reported stimulatory effects (136) as well as inhibitory effects (57, 58) upon the enzyme. Similar discrepancies were noted when the divalent metal ions were tested (27, 136).

Magnesium was generally found to be inhibitory to the phosphatidate phosphohydrolase (1, 16, 28, 141, 148). Sedgwick and Hubscher (136, 137), however, showed that Mg^{2+} markedly stimulated the enzyme activity at low concentrations, although strong inhibition was observed at high concentrations.

Working with erythrocyte membrane, Hokin et. al. (57, 58) reported the presence of two types of phosphatidate phosphohydrolase activity; one was Mg^{2+}-independent while the other Mg^{2+}.dependent.

Recently, Jamdar and Fallon (68) observed that in the presence of Mg^{2+}, adipose tissue microsomes were more active than cytosol in utilizing membrane-bound PA to form 1,2-DG. In a subsequent investigation (69), the properties and subcellular distribution of the PA-phosphohydrolase were studied. Its activity was assayed with both aqueous and membrane-

bound phosphatidate. When aqueous PA was used as the substrate, its activity was detected in mitochondrial, microsomal, and soluble fractions. While the effect of Mg^{2+} was tested, it was found that it was inhibitory at high concentrations, at low concentrations, however, Mg^{2+} stimulated activity in the microsomes and the soluble fraction. No activity was demonstrated with membrane-found substrate in the absence of Mg^{2+}, but in the presence of Mg^{2+}, the enzyme from soluble and microsomal systems was found to be active against this substrate. Thus, the veracity of the presence of two types of PA-phosphohydrolase seems to be palatable.

3. Soluble Phosphatidate Phosphohydrolase. It was generally believed that the phosphatidate phosphohydrolase activity was localized in the subcellular particulate fractions. Using aqueous phosphatade dispersion, only about 10% of the enzyme activity was found in the particle-free supernatant. It was not until 1967 that the presence of the soluble phosphatidate phosphohydrolase was demonstrated.

In 1957, Stein and Shapiro (143) observed that the biosynthesis of neutral glycerides via the sn-glycerol 3-phosphate pathway in the mitochondrial fraction of rat liver homogenates was greatly stimulated by a supernatant fraction. These experiments have since been confirmed and extended in several laboratories (19, 20, 31, 62, 72, 133, 139, 140, 151).

Stein et. al. (144) discovered that the active principles in the particle-free supernatant consisted of two factors, one was heat-stable and the other heat-labile. They further showed that the heat-stable factor could be partly extracted with ethanol-ether. The heat-labile factor was shown through later investigations performed by various workers to consist of several components.

Brindley, Sedgwick and Hubscher (20) presented evidence indicating that the heat-stable factor in the supernatant was unsaturated fatty acids. Using rat liver mitochondria or microsomal fraction of cat intestinal mucosa, they found that the biosynthesis of glycerides was stimulated by the addition of unsaturated long chain fatty acids, especially linoleate and linolenate.

To prove that the stimulatory effect of the supernatant was at least partly due to the presence of unsaturated fatty acids, they removed the free fatty acids from $(NH_4)_2SO_4$- precipitated particle-free supernatant by acetone treatment. The acetone treated $(NH_4)_2SO_4$- precipitate particle-free supernatant was found to give a lower stimulation than did the original preparation. This loss in stimulating activity was shown to be completely restored by adding $C_{18:2}$. The free fatty acid composition of the supernatant was also analyzed. The data showed that 43% of the free fatty acid consisted of unsaturated C_{18} acids. Although it was noted

that the stimulating effect of unsaturated fatty acids was small in comparison to that of the whole supernatant fraction, the participation of fatty acids in the active principle was evident.

The heat-labile factor was found to be inactivated after heating at 70C for 3 minutes. It was found to travel as a relatively sharp band with the protein front on a column of Sephadex G-200, indicating a molecular weight of 200,000 or more by Smith and Hubscher (139). These authors were also able to effect a 3 to 6 fold purification by precipitating the stimulating factor with ammonium sulfate. The precipitate was found to be destroyed by treatment with papain, indicating the protein nature of this factor.

Glyceride biosynthesis has been shown to be activated by the addition of albumin and/or lipoproteins (31, 63, 139, 151). It was also reported that the stimulatory effect of lipoproteins diminished when they were defatted (151). In view of the stimulating effect of unsaturated fatty acids, it is possible that the presence of such fatty acids was, at least, part of the cause of the lipoprotein stimulation.

Palmityl CoA or palmitate were generally used in the glyceride biosynthesis experiments. It is known that palmityl CoA and free fatty acids are inhibitory to various enzyme systems (142, 149) and that such an inhibitory effect can be alleviated by the addition of albumin. It is possible

that the heat-labile factor in the supernatant exerts its effects similar to that of albumin and/or various lipoproteins. However, the much greater activity of the supernatant fraction, together with the fact that it was ineffective when glyceride biosynthesis was operating via the monoglyceride pathway, make it seem unlikely that the stimulation can be ascribed to a non-specific protein.

Evidence indicating that the stimulating effect of the supernatant fraction upon the biosynthesis of triglyceride via the sn-glycerol-3-phosphate pathway was due mainly to the presence of L-α-phosphatidate phosphohydrolase was first presented by Johnston and co-workers (72) and Smith and associates (140). These workers found that in the absence of the supernatant fraction, phosphatidic acid was the main product synthesized by the microsomal and mitochondrial particulate enzymes. Triglyceride synthesis was greatly stimulated by the addition of the particle-free fraction. The increase in TG production was paralleled by a decrease in phosphatidic acids. Furthermore, the total incorporation of radioactive substrates under the stimulated or unstimulated conditions was found to be nearly at an identical level varying only in the distribution of labeled substrate between phosphatidic acids and triglycerides. The authors concluded that the stimulating effect was localized in the biosynthetic steps following phosphatidic acid

formation. The main function of the supernatant therefore appears to be to supply the enzyme L-α-phosphatidate phosphohydrolase for the conversion of phosphatidic acids to 1,2-diacyl-sn-glycerol which in turn serves as a precursors of triglycerides and possibly phospholipids.

It has been shown that the soluble enzyme was effective in hydrolyzing the membrane-bound biosynthetically-produced phosphatidates but not the aqueous dispersion of phosphatidates, while the particulate phosphatidate phosphohydrolase was active against the soluble PA only (72, 140). Recent findings (69) indicate that the enzyme activity could be detected in the mitochondria and in the microsomes as well as in the soluble fraction when aqueous PA was used as the substrate. Furthermore, the rate of utilization of aqueous PA was found to be always higher than that of membrane-bound substrate.

4. Functions of Phosphatidate Phosphohydrolase. Although the role of the particulate phosphatidate phosphohydrolase in lipid biosynthesis has been suggested (27, 62, 141, 143, 144, 148, 160), Wilgram and Kennedy (163) reporting on studies of the subcellular distribution of this enzyme, reported that its location was very different from that of diglyceride acyltransferase and choline-phosphotransferase which catalyze the reactions that utilize the products of the phosphohydrolase reaction. Its occurrence in the

sub-cellular fractions devoid of other α-GP enzyme activities was also reported by Brindley and Hubscher (19). It is thus possible that the soluble enzyme is the one involved in lipid biosynthesis while the particulate enzyme is engaged in other functions.

The involvement of the particulate phosphatidate phosphohydrolase in the active transport of sodium and potassium has been suggested by various investigators. Coleman and Hubscher (27) studied the distribution of this enzyme in the liver, kidney, brain, and intestinal mucosa of the ox, pig, rabbit, guinea pig, and rat. They found that the specific activity of the enzyme was higher in kidney and intestinal mucosa than in the brain and liver. Since these two organs are known to engage actively in transport, such a finding is consistant with the suggested role of this enzyme in active transport. Hokin and Hokin (56) reported that the incubation of slices of salt gland of the albatross in a system where $Na^+ + K^+$ transport was enhanced showed increased incorporation rates of ^{32}p into phosphatidic acid and ^{32}p and inositol-2-^{3}H into phosphoinositide. A scheme, termed the phosphatidic acid cycle, in which sodium transport is coupled to the renewal of phosphate in PA was proposed. The reported occurrence of PA-phosphohydrolase in liver cell surface membrane (25) and erythrocyte membrane (57, 58) as well as that of high ($Na^+ - K^+$)- coupled ATPase activities in a

purified surface membrane preparation (36) tend to support this view.

The hydrolysis of a number of mono alkyl phosphates (1, 37), 1,3-diacylglycerol-2-phosphate (58), and lysophosphohydrolase activity have been reported in a number of systems. It is possible that phosphate compounds with lipid-like properties could act equally well as substrate for PA-phosphohydrolase and could be used in the metabolism of the molecules which compose the surface of the cell. The possibility that the particulate enzyme is involved in the synthesis-resynthesis of membrane phospholipids in growth also cannot be ignored. However, the nature of the true functions and true substrates of this enzyme needs further investigations.

Observing the different distributions and fluctuations of the activity of phosphatidate phosphohydrolase in various neural tissues, McCaman et. al. (111) speculated that such variations might be due to a feedback mechanism responsive to the glycerophosphatide content of the tissues involved. Recently, Vavrecka, Mitchell, and Hubscher (156) presented evidence indicating that the phosphatidate phosphohydrolase enzyme may be rate-limiting in the biosynthesis of glycerides via the sn-glycerol-3-phosphate pathway. A significant increase in its activity upon starvation and decrease again after refeeding were also shown. It thus seems feasible

that this enzyme may have a regulatory function in TG synthesis. The validity of such assumptions awaits substantiation and the mechanism of regulation requires further studies.

IV. Role of Diglyceride in Lipid Metabolism

1. In Vivo and in Vitro Evidence Indicating 1,2-diacyl-sn-glycerol as a Direct Precursor for Tri-glyceride Formation. Kinsella (86) in a study of the incorporation of ^{14}C-glycerol into lipids by dispersed bovine mammary cells found that the $^{14}C_3$-glycerol present in the culture medium was actively absorbed and metabolized by these cells. When the percentage of distribution of radio activity among the lipids was studied, the specific activity time curves tentatively indicated that the triglycerides were derived from the 1,2-diacyl-sn-glycerols.

In vitro evidence indicating that the final step in the biosynthesis of triglycerides in liver is the esterification of 1,2-diglycerides by activated fatty acids has been provided by Weiss and Kennedy (158). Data in agreement with these findings have been presented by various investigators (143, 144, 156, 159). A precursor role for DG in TG formation in homogenates of rat epididymal fat pads was suggested by Steinberg, Vaughan, and Margolis (146).

Fatty acylCoA:diglyceride acyltransferase activity was

detected in particulate fractions of cell-free preparation of E. coli (117). The subcellular distribution of this enzyme in animal tissues has been studied by a number of researchers. Wilgram and Kennedy (163) studying the intracellular distribution of several lipid synthesizing enzymes in rat liver reported that the activity of DG-acyltransferase was confined to the microsomal fraction. These findings were also corroborated by Stein et. al. (143). Brindley and co-workers (20) and Johnston and colleagues (72).

Using ^{14}C-palmitylCoA and 1,2-diglyceride as substrates, Smith and Hubscher (139) detected DG-acyltransferase activity in a mitochondrial preparation from rat liver. Stein, Tietz, and Shapiro (144) reported similar results. These authors also provided evidence that the proportion of lower glycerides to triglycerides was much higher in the microsomes than in the mitochondria, and upon the addition of mitochondria to the microsomal system, a shift of the lower glycerides to triglycerides was observed.

2. The Specificity of the Esterification of 1,2-diacyl-sn-glycerol. There are three generally accepted hypotheses concerning the fatty acid positional distribution within the triglyceride molecules of natural fats: 1. the random distribution, 2. the restricted random, or 1,3-random, 2-random, and 3. the stereospecific distribution hypotheses.

It has been found that certain catagories of fatty acids

do tend to concentrate at a certain position of the glycerol molecules in the natural fats. For example, in terrestrial animal fats, the C_1, C_2, and C_3 of the glycerol molecule are occupied predominantly by saturated, monoenoic, and polyenoic acids, respectively. In marine animals and fish, long-chain polyunsaturated fatty acids tend to concentrate at carbon number two. Short chain fatty acids are generally more often found to accumulate at the 3-position of the glyceride. Examples of such analyses are the studies of Hanahan et. al. (52), Mattson and Beck (104), and Youngs (165) on animal fats, and Walker et. al. (157) on the lipids of microorganisms.

Some evidence has been presented that the stereospecific distribution of fatty acids on the triglyceride molecules may be due to the selectivity of the DG-acyltransferase.

Goldman and Vagelos (42) studying the esterfication of DG to TG in the particulate enzyme system obtained from chicken adipose tissue, have found that DG with at least one unsaturated fatty acid was more reactive, in general, than saturated diglycerides as a substrate. When the reactivity of various DG was compared, these authors reported that DG containing myristate were more active than those containing palmitate, which in turn were more reactive than those containing stearate.

3. 1,3- or 1,2-diacyl-sn-glycerol as the Active Substrate in TG Formation. Clark and Hubscher (25) reported that with enzyme preparations from intestinal mucosa, 1,3-disterarin stimulated the incorporation of ^{14}C-palmitate into TG nearly as well as 1,2-dipalmitin did, suggesting that the esterification is not specific for either the 2- or 3-hydroxyl group of the glycerol molecule.

Brown and Johnston (21) using doubly labeled monopalmitin demonstrated the utilization of intact 1-monopalmitin by intestinal mucusal enzymes in TG synthesis. They further reported that the first acylation occurred at the corresponding primary hydroxyl group forming the 1,3-DG. Evidence indicating that the 1,3-DG was the active substrate in TB biosynthesis was further provided by showing its accumulation in the system when fatty acids become a limiting factor.

It has been found, however, that generally the 1,2-DGs are more commonly used as substrates for the formation of TG in other animal tissues (72, 139, 143, 158, 163). Data indicating that 1,3-diglyceride was unreactive whereas all 1,2-diglycerides tested were reactive in the biosynthesis of TG in chicken adipose tissue were presented by Goldman and Vagelos (42).

It is well established that the predominant pathway for TG formation in the intestinal mucosa is the monoglyceride

pathway. Whether such a discrepancy regarding the precursor role of 1,2- and 1,3-DG in the biosynthesis of triglycerides is due to the presence of different enzyme systems awaits further elucidation.

4. Diglycerides and Phospholipid Biosynthesis. Weiss and Kennedy (158) evolved the concept that neutral glycerides and phospholipids are formed by a similar system proceeding via glycerol phosphates and phosphatidic acid by the following reaction steps:

a. sn-glycerol 3-phosphate + 2 palmitylCoA → PA

b. PA → DG + P_i

c. DG + PalmitylCoA → TG

d. DG + phosphorylcholine → choline-phospholipid

Net synthesis of lecithin was demonstrated when isolated mitochondria from chicken liver were incubated with CDP-choline and 1,2-diglyceride (160). Similar results have been reported when chicken liver and various tissues of rat (141) and rat brain dispersions (148) were used as the enzyme source. Gurr, Brindley, and Hubscher (45) demonstrated the occurrence of CDP-choline: 1,2-diglyceride cholinephosphotransferase in the intestinal mucosa of the cat, guinea pig, and rabbit. The characteristics of the enzyme with respect to substrate concentration, pH optimum, Mg^{2+} requirement, inhibition by Tween-20, and stability to freezing and thawing were studied.

Although it is well established that the lecithin formation in animal tissues is by the condensation of CDP-choline with a diglyceride, it has been found that certain phospholipids in cell-free extracts of E. coli are derived from CDP-DG and activated bases (75). When phosphatidates and cytidine-5-^{3}H-5′-triphosphate were incubated with the cell-free system of mycobacteria, labeled CDP-DG was isolated (Walker, unpublished data). However, whether CDP-DG serves as precursor for the biosynthesis of phospholipids in these organisms or whether the true precursors are CDP-bases and DG, as is the case in the animal tissues, awaits further investigation.

In 1959, Hokin and Hokin (59) discovered a different pathway for phosphatidic acid snythesis. Diglyceride was found to be phosphorylated by ATP by brain microsomes and deoxycholate extracts of microsomes. The enzyme which catalyzes this reaction has been termed DG-kinase. Activity of this enzyme was also detected in bacterial cells by Pieringer and Kunnes (118), and by Chang and Kennedy (23).

It was reported that Mg^{2+} as well as the detergent Cutscum were required for the activity of the DG-kinase, and that it was stable for 3 minutes in a boiling water bath (118). The high degree of stereospecificity of this enzyme in the phosphorylation of DG was described (23), and the substrate specificity has been suggested.

The function of DG-kinase is not clear, Pieringer et. al. (117) suggested that its role may possibly be to scavenge glycerides formed by degradative processes, converting them back to phospholipids. Hokin and Hokin (56, 59) presented evidence indicating that sodium transport is coupled to the renewal of phosphate in PA and that this renewal is catalyzed by the combined action of DG-kinase and PA-phosphohydrolase. This view was supported in further studies by Hokin, Hokin, and Mathison (58).

MATERIALS AND METHODS

1. Organism. Mycobacterium smegmatis ATCC strain 19420 was grown in 2.8 liters Fernback flasks containing 1 liter of modified Youman's medium containing either 2% glucose or 4% glycerol (157). The culture was incubated at 27C for two to four days (referred to as young and old cells, respectively) on a rotary shaker. The cells were harvested by centrifugation before used.

2. Media.

a. Modified Youman's glycerol medium. The composition of this medium is:

	Per liter
KH_2PO_4	5.0 g
Glycerol	40.0 g
Asparagine	5.0 g
Sodium Citrate	2.5 g
Ferric Ammonium Citrate	0.05 g

0.5 g Magnesium sulfate was added after the pH of the above mixture was adjusted to 7.0.

b. Modified Youman's glucose medium. The basic formula of this medium is the same as that of the glycerol medium except that glycerol was replaced by 2% glucose.

The glucose was sterilized and added separately to each flask prior to the inoculation of the organism.

3. Materials. ^{14}C-oleate, ^{14}C-glycerol, and other radioisotopes used in this study were purchased from Applied Science, Amersham-Searle, ICN, or Tracer Laboratories. Chromatographically pure $^{D}/_{L}$ ^{14}C- α-glycerophosphate was prepared in our laboratory. Other reagent grade compounds were purchased from Calbiochem, Fisher, or P-L Laboratories. Analytical grade aldolase (12 E.U./mg Protein), Triophosphate isomerase (3,100 E.U./mg Protein), Glycerokinase (90 E.U./mg Protein), α-glycerophosphate dehydrogenase (134 E.U./mg Protein) and Glyceraldehyde dehydrogenase (81 E.U./mg Protein) were purchased from Sigma Chemical Company.

4. Preparation of Cell-Free Extracts.

a. Total Cell-Free Extract. Cells of desired phase of growth were harvested by centrifugation at 12,000 xg for 15 minutes. After washing in 0.05M Tris (Hydroxymethyl) Aminomethane·HCl (Tris·HCl) buffer pH 7.5 (containing 10^{-3}M dithiothreitol, DTT), cells were disrupted by two passages at 20,000 psi through a French pressure cell (American Instrument Company, Silver Springs, Maryland). Cell debris and unbroken cells were removed by centrifuging at 6,000 xg for 15 minutes. The supernatant fraction thus recovered was referred

to as the total Cell-Free Extract (CFE).

b. Fractionation of the Total Cell-Free Extract into cytosol and membrane fractions. The cytosol and membrane fractions were obtained by centrifuging the total Cell-Free Extract at 37,000 xg for 1 hour. The pellet (membrane) fraction was resuspended in 0.05 M Tris·HCl buffer pH 7.5 containing 10^{-3}M DTT (unwashed pellet). In some experiments, the pellet fraction was washed once with 10-15 volumes of the above buffer, recovered by centrifugation at 37,000 xg for 40 minutes prior to use (washed pellet).

c. Ammonium Sulfate Salt Fractionations. In some cases, the supernatant (cytosol) proteins were further separated into various fractions by making the supernatant to 0-20%, 20-40%, 40-60%, and 60-100% saturation with ammonium sulfate. The desired amount of the salt was added to the supernatant fraction slowly with stirring. After all of the salt had dissolved, the solution was placed at 4C and allowed to stand for half an hour. The precipitated proteins were collected by centrifuging at 10,000 xg for 15 minutes.

In certain experiments, the nucleoproteins in the supernatant fraction were removed with $MnSO_4$

prior to the ammonium sulfate fractionation. The supernatant was made 0.0235 M with respect to $MnSO_4$ and allowed to stand at 4C overnight. The supernatant solution obtained by centrifuging at 30,000 xg for half an hour was referred to as the "nucleoprotein-free supernatant" fraction and was used for ammonium sulfate fractionation.

d. Ultrafiltration. The supernatant (cytosol) proteins of different range of molecular weights were separated from each other by ultrafiltration with a Stirred Ultrafiltration cell (Amicon Corporation, Lexington, Mass.) using various Diaflo membranes (xM300, xM100A, and xM50). The flow rate and other conditions used were as recommended by the manufacturer.

5. Protein Determination. The protein concentrations of the Cell-Free Extracts were determined by one of the following methods. Bovine serum albumin (BSA) was used to obtain the standard curve for these processes.

a. Biuret Method (132). The biuret reagent was prepared by making 1.5g $CuSO_4.5H_2O$, 6.0g $NaKC_4H_4O_6$, and 10% carbonate free NaOH into one liter solution.

One ml of the sample was mixed with four ml biuret reagent. After standing at room temperature for 30 minutes, the optical density of the

mixture was determined at 550 nm with a Spectronic 20 with BL cells (Ca.1.1 cm path).

The protein content of the sample was determined by comparing its O.D. with that of the standard curve.

b. Modified Biuret Method. Pellet protein concentration was determined by a modified biuret method. The membrane-bound protein was digested by mixing 2.5 ml sample with 1.25 ml 3 N NaOH and heating the mixture to boiling for 5 minutes. After cooling to room temperature, 1.25 ml of 2.5% $CuSO_4.5H_2O$ was added to the mixture and allowed to stand for 5 minutes. The sample was then centrifugated and its optical density read at 555 nm in a Spectronic 20 with BL cells.

c. Modified method of Lowry et. al. (101). The following reagents were prepared for protein determination by this method.

A) 2% Na_2CO_3 in 0.1 N NaOH

B) 0.5% $CuSO_4 \cdot 5H_2O$ in 1% Na- or K - Tartrate

C) Freshly mixed 50 ml A and 1 ml B prior to use.

D) Diluted Folin (titrated Folin phenol reagent with NaOH to phenophthalein end point, diluted Folin about 2 x to make I N acid).

Protein in solution. 1 ml C was added to 200µl of sample (containing 5-100 µg protein) in a 3-10 ml test tube. After standing at room temperature for 10 minutes, 100µl D was added rapidly to the mixture and mixed in 1-2 seconds. The sample was allowed to stand at room temperature by an additional 30 minutes. The optical density of the mixture was then read at 750 nm (5-25 µg Protein/ml) or at 500 nm (stronger solution).

Pellet protein. Pellet protein was digested prior to its determination by the above procedure. To 5-100 µg precipitated protein, 0.1 ml I N NaOH was added. The mixture was then heated at 100C for 10 minutes.

6. Enzyme Assays.

a. Glycerol dehydrogenase. The $NADP^+$ and NAD^+ specific glycerol dehydrogenase were assayed according to the method of Goldman (43). The assay mixture was as follows:

	mM
2-amino-2-methyl-1,3-propanediol buffer (pH 9.1)	80
Glycerol	200
L-cysteine	5
$NADP^+$ or NAD^+	1

0.5 to 2 mg of protein was added to the above

mixture. After incubating at 35C for 30 minutes, the absorbancy at 340 mμ was measured.

One unit of enzyme is defined as that amount of enzyme which catalyzes the reduction of 1.0 μmole of $NADP^+$ or NAD^+ under the assay condition.

b. α-glycerophosphate dehydrogenase. This enzyme was assayed according to the method of Beisenherz, Bucher, and Garbade (14). The assay mixture for this enzyme is shown below:

DPNH	1.35×10^{-4} M
Dihydroxyacetone phosphate	1.0×10^{-4} M
Triethanolamine-HCl buffer (pH 7.5)	5.0×10^{-2} M

The amount of DPNH stock solution required to give an absorbance of 0.75 at 366 mμ in the assay mixture was determined by a preliminary experiment. The assay was started with this absorbance. After adding the enzyme preparation to the mixture, the reaction was allowed to proceed for 30 seconds. The time required for a change in absorbance of 0.1 was then determined.

One unit of enzyme is defined as follows:

$$U = \frac{100}{\text{Seconds for change of absorbance of 0.1}}$$

c. Aldolase. Aldolase was assayed by the method

described by Taylor (150).

Reagents:

Fructose 1,6-diphosphate (FDP)	0.1 M
Sodium Arsenate	0.17 M
DPN	0.005 M
Glycine	0.27 M
Cysteine	0.1 M
D-glyceraldehyde-3-phosphate dehydrogenase	2.5 mg Prot./ml

Into a spectrophotometer cuvette was placed 0.5 ml of FDP, 0.3 ml of arsenate, 0.1 ml of DPN, 0.3 ml of glycine, 0.6 ml of cysteine, 1.0 ml of H_2O, and 0.1 ml of dehydrogenase. After incubating for 10 minutes, 0.1 ml of enzyme solution was added to the mixture and the optical density at 340 mμ was recorded at 30 second intervals.

O.D in 1 minute/6.22×10^6 = Moles FDP transformed/minutes.

Moles FDP/min. x 2 x 31 x 10^3 = Mg P transformed/minutes.

One unit of enzyme is defined as that amount which transformed 1 mg P in 1 minute.

d. Triose phosphate isomerase. Triose phosphate isomerase was assayed according to the method described by Beisenherz (13).

The assay mixture contained:

DPNH	8.5×10^{-5} M
DL-glyceraldehyde-3-phosphate	3.0×10^{-4} M
Triethanolamine-HCl buffer (pH 7.9)	2.0×10^{-2} M
α-glycerophosphate dehydrogenase	8 µg/ml

The reaction was started by adding the enzyme preparation to the mixture. After allowing the reaction to proceed for a short while, the time required for the absorbance to decrease by 0.1 at 366 mu was recorded.

One unit was defined with the same units as used for α-glycerophosphate dehydrogenase.

e. Glycerol kinase. Glycerol kinase activity was demonstrated by incubating the enzyme preparation with 60 umoles of Tris·HCl buffer, pH 8.7, 6 umoles of $Mgcl_2$, 3 umoles of ATP, and 150 umoles of glycerol in a final volume of 3 ml. The incubation was carried out at 35C for 1 hour. The reaction was stopped by freezing.

The amount of α-glycerophosphate formed under the above conditions was assayed by the method of Hohorst (55).

Reagents:

Hydrazine-glycine buffer (0.5M hydrazine; 1M glycine; pH 9.5)

DPN — Ca. 5 x 10^{-2}M β-DPN

α-glycerophosphate dehydrogenase — Ca. 6 mg Protein/ml

To 0.5 ml of the incubation mixture, 0.45 ml of hydrazine-glycine buffer and 0.05 ml DPN solution were added. After mixing, the mixture was allowed to stand and reach room temperature. The initial optical density, E, was read twice at an interval of 3 minutes at 340 mμ (1 cm light path), 0.01 ml α-glycerophosphate dehydrogenase solution was then added to the mixture. Ten to twenty minutes after the enzyme addition, the O.D. E_2 was read twice at an interval of 3 minutes.

The amount of α-glycerophosphate in the sample was calculated from the optical density difference, ΔE as follows:

$$\Delta E \times F = \mu\text{moles } \alpha\text{-GP/g Protein}$$

$$F = \frac{dil}{\varepsilon \times d}$$

dil = total dilution of the sample

ε = extinction coefficient (cm^2/umole)

d = light path (cm)

To check if the various assay systems were working correctly, analytical grade enzymes purchased from commercial sources were used in place of the cell free preparations.

Zero time reaction as well as boiled enzymes were run as controls for the foregoing assay procedures.

7. Glass Filter Paper Disk Assay. In testing the ability of the various fractions (Total Cell-Free Extract; Supernatant and Pellet fractions of the Cell-Free Extract, and the various fractions of ammonium sulfate precipitated supernatant proteins) to incorporate ^{14}C-glycerol or ^{14}C- α-glycerophosphate into lipids, the Glass Filter Paper Disk method was used. The various fractions were incubated with the labeled substrates and other co-factors under the conditions tested (see Table 34). At the end of incubation, 100 μl of the mixture was dried on a Glass Filter Paper Disk with warm air for 15 seconds. Non-lipid materials were washed away with cold, Trichloroacetic acid (10%-15 seconds, 5%-30 minutes, 1%-2x, 10 minutes each). The disk was dried with hot air for 30 minutes prior to the measurement of radioactivity.

8. Thin Layer Chromatography. Adsorbosil-1 ($CaSO_4$ binder, Applied Science Laboratories, Inc., University Park, Pennsylvania) and Adsorbosil-3 ($MgSiO_4$ binder, same source) were used for the isolation of neutral lipids and polar lipids, respectively.

Plates were prepared by suspending the gel in water and spreading the slurry on 20 x 20 cm glass plates at a thickness of 0.05 mm. The plates were activated at 120C for 1½

and stored in a dessicator.

Sample and a standard were spotted about one inch from the bottom of the plate. For the separation of neutral lipids, the adsorbosil-1 plate was developed in hexane: ether: acetic acid (70: 30: 3 by vol.). Lipids were located by comparing with the standard after spraying the plate with rhodamine 6-G and viewed under ultra violet light.

Polar lipids were separated on adsorbosil-3 plates which were developed first in ether. After the solvent had reached the top of the plate, the plate was taken out, dried, and redeveloped in chloroform: methanol: Formic acid: water (80: 20: 8: 0.3 by vol.). The solvent was allowed to travel up 4/5 of the plate. A standard was routinely spotted on one side of the plate and was sprayed with Vaskovsky and Kostetsky spray (154). Lipid corresponding to the standard were located under UV light after spraying with rhodamine 6-G.

9. Experiment with Cell-Free Extracts. The various conditions and co-factors for the cell-free experiments were covered in Results. Experimental reactions were interruped by adding 2 volumes of chloroform: methanol (2:1) containing 0.6% 2 N HCl. Lipid materials were extracted twice with the same solvent system. After mixing and centrifugation, the lower layer containing the lipid materials was aspirated carefully to another tube. The combined

solvent was dried slowly under a stream of nitrogen. 100 µl Folch solution containing standard markers (TG, DG, MG, PA) was added. Generally a 10 µl aliquot was taken for counting and 40 µl was spotted on thin layer chromatography plates.

10. Measurement of Radioactivity. A 2-channel liquid scintillation counter (model 500D, Packard Institute Company, Inc., LaGrange, Illinois) was used for the measurement of radioactivity.

The Glass Filter Paper Disk or the different lipid classes scraped from the TLC plates were placed in scintillation vials. Five-ten mls modified Bray's (18) solution were added to the vial and mixed thoroughly. Samples were counted for 10 - 30 minutes.

RESULTS

I. Enzyme Assays

Logarithmic phase Mycobacterium smegmatis cells grown in modified Youman's glucose or glycerol medium were harvested and washed twice with 0.05M Tris buffer pH 7.5 containing 10^{-3}M Dithiothreitol. Washed cells were re-suspended in the same buffer system. Cell-Free Extract was prepared and fractionated into supernatant (cytosol) and pellet (membrane) fractions. Procedures for such processes are covered in Materials and Methods.

The specific activities of a number of selected enzymes of these preparations were tested according to scientifically accepted biochemical methods as described in Materials and Methods. Results are shown in Table 1.

It is evident that activities of glycerol dehydrogenase, glycerol kinase, and aldolase are present in the supernatant fraction only. However, that of α-glycerophosphate dehydrogenase was detected in both the supernatant and pellet fractions, although the specific activity of this enzyme in the latter fraction was only about one half that of the supernatant fraction.

The triose phosphate isomerase activity of both the supernatant and pellet fractions was also assayed. No

Table 1

The Specific Activities of Selected Enzymes in Supernatant and Pellet Cell-Free Extract Fractions of Glucose and Glycerol Grown M. Smegmatis Cells

	Units/mg Protein				n moles GP formed per mg Protein per hour			
	Glycerol dehydrogenase		α-glycerophosphate dehydrogenase		Aldolase		Glycerol Kinase	
	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet
Glycerol grown	0.028	0	1.6	0.8	3.3×10^{-4}	0	43	0
Glucose grown	0.036	0	0.15	0.31	4.5×10^{-3}	0	20	0

Conditions: 30,000 xg/1 hr. supernatant and washed pellet suspended in 0.05M Tris buffer p^H 7.5 containing 10^{-3}M DTT were used. Assay procedure and definition of units are covered in Materials and Methods.

conclusive data could be obtained using the assay method of Beisenherz (13). Circumstantial evidence was obtained in some other experiments while assaying the aldolase enzyme that this enzyme was present in the supernatant fraction of both glucose and glycerol grown cells. Because of vagaries of the assay method, consistent unit measurements could not be obtained.

When the specific activities of the selected enzymes in Cell-Free Extracts of glycerol grown cells were compared to those of the corresponding enzymes in preparation of glucose grown cells, no significant difference in glycerol dehydrogenase activity was observed. The NAD^+- or $NADP^+$-dependency of this enzyme was also tested. It was found that the enzyme could utilize NAD and NADP as hydrogen-carrier to the same degree of efficiency.

The activity of α-glycerophosphate dehydrogenase was found to be nearly three times higher in glycerol cells than that in glucose cells. This is probably due to the fact that when glycerol is utilized as the main carbon source, a considerable amount of α-glycerophosphate has to be oxidized to dihydroxyacetone phosphate to enter the glycolytic pathway for energy production as well as for its metabolism into other materials necessary for the snythesis of cellular components. Higher specific enzymatic activity due to the adaptation to the availability of substrate was

also observed when aldolase and glycerol kinase in cells grown in the two different media were assayed.

II. Incorporation of ^{14}C-oleate into Lipids by Supernatant and Pellet Fractions of M. Smegmatis Cell-Free Extract.

In preliminary studies it was found that the incorporation of ^{14}C-oleate into lipids by Cell-Free Extracts of M. smegmatis was enhanced by certain glycolytic products. In an attempt to elucidate the true effect of these compounds on lipid biosynthesis, a number of such substrates were incubated, together with other necessary co-factors for lipid formation, with total Cell-Free Extract, and with the 30,000 x g/1 hour pellet and supernatant fractions prepared from Cell-Free Extract of M. smegmatis grown in modified Youman's glycerol or glucose medium.

1. Effect of Triose Phosphates on ^{14}C-oleate Incorporation into Lipids by Total Cell-Free Extract and Supernatant Fraction of Cell-Free Extract. In the following experiments, 4-6 mg of total Cell-Free Extract of supernatant protein, suspended in 0.05 M Tris buffer pH 7.5 containing 10^{-3}M DTT, was incubated with 100 µmoles of phosphate buffer pH 7.55, 5 µmoles each of ATP, Mg^{2+}, NaF, mercaptoethanol, and 0.05 µmole of CoA in a total volume of 1 ml. The incorporation of ^{14}C-oleate, and the effects of triose phosphates on its incorporation into lipids by the cell-free systems were studied.

A noticeable difference in the effect of glyceraldehyde-3-phosphate was observed. There was no appreciable change in the incorporation of ^{14}C-fatty acid into lipids by both the supernatant and total Cell-Free Extract from glucose cells when the preparation had been made to 5 mM with glyceraldehyde-3-phosphate (Table 2). On the other hand a considerable increase in the incorporation of labeled substrate into lipids was observed when supernatant fraction from glycerol grown cells was used. A substantial portion of the label was found to be present in the triglyceride fraction (Table 3).

On the other hand, when the effect of α-GP on lipid biosynthesis was examined, a similar enhancement in the incorporation of ^{14}C-oleate into polar lipids with a decrease in radioactivity in the triglyceride fraction was observed. A more prominent effect of this substrate with the supernatant fraction of glucose cells compared to the glycerol cells was observed.

The differences observed between the effect of added glyceraldehyde-3-phosphate and α-glycerophosphate upon Cell-Free Extracts from glycerol and glucose grown cells may reflect the differences in the intracellular concentrations of these compounds. Experiments, reported later in this study, were performed in an attempt to resolve the nature of these differences.

Table 2

Incorporation of ^{14}C-oleate into Lipids by Total Cell-Free Extract* and Supernatant Fractions Prepared From Glucose Grown Young** M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Total CFE	213,000	100	62,500	88	2,400	3.4	6,200	8.7
Total CFE + 5 µm GA3P	203,000	100	54,700	81	3,990	5.9	9,100	13
Total CFE + 5 µmαGP	242,000	100	58,100	72	7,700	9.5	15,000	19
Supernatant	220,000	100	62,000	85	3,100	4.2	8,200	11
Supernatant + 5 µm GA3P	219,000	100	61,000	83	3,700	5.1	8,400	12
Supernatant + 5 µmαGP	214,000	100	49,700	70	7,000	9.8	14,500	20

Conditions: 4-6 mg Protein, oleic acid (sp. act. 10 µCi/µmole) 441,000 dpm. phosphate buffer, pH 7.55 100 µmoles, 0.05µmole CoA, 5 µmoles ATP, 5 µmoles Mg^{2+}, 5 µmoles mercaptoethanol, 5 µmoles NaF, in a total volume of 1 ml. 1 hr. incubation at 35C.

* preparation of cell-free extract discussed in Methods and Materials

** early log-phase cells

Table 3

Incorporation of ^{14}C-oleate into Lipids by Supernatant Fractions Prepared From Glycerol Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Supernatant	128,000	100	94,000	74	17,800	14	16,000	13
Supernatant + 5 µm glyceraldehyde 3-phosphate	248,000	100	187,000	75	35,000	14	26,000	11
Supernatant + 3 µm 1,2-diphospho-glycerol	254,000	100	206,000	81	21,000	8	27,000	11
Supernatant + 3 µm 1,3-diphospho-glycerol	260,000	100	213,600	82	19,000	8	27,000	11
Supernatant + 5 µm -glycero-phosphate	140,000	100	95,000	68	20,500	15	24,000	17

Conditions: same as Table 2

2. Effect of Triose Phosphates on the Incorporation of ^{14}C-oleate by the Lipids of Pellet Fractions of M. Smegmatis Cell-Free Extracts. Previous experiments have shown that certain enzymatic activities involved in lipid synthesis were not detected in the 30,000 xg pellet fraction of M. smegmatis Cell-Free Extract (Table 1). In order to determine if the pellet fraction could incorporate labeled fatty acids, this fraction was incubated with the necessary co-factcrs at 35C for one hour. The effects of various triophosphates, namely, 1,2- and 1,3-diphosphoglycerols, glyceraldehyde-3-phosphate, α-glycerophosphate, 2-, 3-phosphoglycerate and 2,3-diphosphoglycerate, on ^{14}C-oleate incorporation into lipids by the pellet fraction was also studied.

With the exception of α-glycerophosphate, all of the triose phosphates tested appeared to cause an enhancement of incorporation of labeled oleate by the lipids, particularly by the triglyceride fraction (Tables 4 to 6). α-glycerophosphate, on the other hand, stimulated incorporation only slightly cr not at all. With this compound, however, a marked shift in incorporation from the neutral lipids to the polar lipids was observed (Tables 4, 5 and 7). This shift has been shown to be due to the formation almost exclusively of phosphatidic acid in the polar fraction.

In studying the effect of glyceraldehyde 3-phosphate on lipid formation in supernatant fraction of glucose and

Table 4

Incorporation of ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glycerol Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	88,700	100	52,200	59	10,200	11	26,400	30
Pellet + 5 μm glyceraldehyde 3-phosphate	219,000	100	120,400	55	15,600	7	83,200	38
Pellet + 3 μm 1,2-diphospho-glycerol	232,000	100	157,300	68	18,700	8	56,000	24
Pellet + 3 μm 1,3-diphospho-glycerol	253,000	100	163,000	64	25,000	10	65,600	26
Pellet + 5 μm α-glycero-phosphate	133,000	100	37,700	28	20,500	16	74,000	56

Conditions: same as Table 2
Pellet (30,000 xg/1 hr.) unwashed

Table 5

Incorporation of ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	140,000	100	123,300	88	7,050	5	9,160	7
Pellet + 5 μm glyceraldehyde 3-phosphate	198,000	100	172,000	87	11,500	6	14,500	7
Pellet + 3 μm 1,2-diphospho-glycerol	274,000	100	251,000	92	8,800	3	14,000	5
Pellet + 3 μm 1,3-diphospho-glycerol	267,000	100	242,000	91	8,400	3	16,200	6
Pellet + 5 μm α-glycero-phosphate	140,000	100	56,400	39	17,800	12	69,700	48

Conditions: same as Table 2

Pellet (30,000 xg/1 hr.) unwashed

Table 6

Incorporation of ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	227,000	100	191,000	84	7,700	3	28,000	13
Pellet + 3 μm 2-phosphoglycerate	262,000	100	225,000	86	5,200	2	32,000	12
Pellet + 3 μm 3-phosphoglycerate	278,000	100	240,000	86	5,800	2	32,000	12
Pellet + 3 μm 2,3 diphosphoglycerate	255,000	100	218,000	85	7,100	3	30,000	12

Conditions: 0.1 μmole CoA used, other conditions same as Table 2

Table 7

Effects of α-glycerophosphate on the Incorporation of ^{14}C-oleate Into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	227,000	100	191,00	84	7,700	3	28,000	13
Pellet + 0.5 μm α-glycerophosphate	150,000	100	77,800	52	19,000	13	53,000	35
Pellet + 1.0 μm α-glycerophosphate	136,500	100	58,000	42	21,000	15	58,000	43
Pellet + 3.0 μm α-glycerophosphate	187,500	100	65,000	35	29,600	16	92,700	50
Pellet + 5.0 μm α-glycerophosphate	164,100	100	50,000	31	25,000	16	89,000	54

Conditions: same as Table 6

glycerol grown M. smegmatis Cell-Free Extract, a conflicting result was observed (Tables 2 and 3). In these experiments results clearly indicate that this compound, like the other glycolytic products, does stimulate fatty acid uptake into lipids. Scrutinizing the data closely reveals the fact that the stimulatory effect of all the triose phosphates is more marked in pellet fractions of Cell-Free Extracts of glycerol cells than in the same fractions of glucose cells. It is possible that the lack of effect of the added glyceraldehyde 3-phosphate in enhancing lipid synthesis in total Cell-Free Extract and in supernatant fraction of Cell-Free Extract of glucose grown cells was due to the presence of large amounts of endogenous glycolytic products in these preparations.

3. Effects of Various Possible Modulators of Lipid Synthesis on ^{14}C-oleate Incorporation into Lipids by Washed 30,000 xg Pellet Fractions of M. Smegmatis Cell-Free Extracts. In an attempt to eliminate the possibility that the stimulatory effects observed in the above experiments were a result of contamination of the pellet fraction by the supernatant enzymes, the pellet fraction was washed with a large volume of 0.05 M Tris buffer pH 7.5 containing 10^{-3}M DTT and centrifuged again at 38,000 xg for an hour. Pellet thus recovered was resuspended in the above buffer system. The ability of the washed pellet

fraction in synthesizing lipids as well as the effects of various possible effects on lipid synthesis was investigated.

It was demonstrated that a considerable amount of labeled fatty acid was incorporated into lipids by the washed pellet fraction of M. smegmatis Cell-Free Extracts. Most of the label (over 80% of ^{14}C-oleate incorporated) was found in triglycerides (Table 8). Evidence is thus presented that if TG are formed by esterifying fatty acyl groups to a preformed acceptor (such as DG or DG derivatives) in the Cell-Free Extract, such an endogenous acceptor is present in the pellet fraction. However, if TG are synthesized de novo from α-GP or other precursors, the enzyme system as well as the precursor can be found in this fraction.

The data presented in Table 8 also indicate the ability of 2,3-diphosphoglycerate, cutscum, and EDTA to stimulate lipid biosynthesis. Although these compounds all enhance fatty acid incorporation, their modes of action are conceivably quite different from each other.

Evidence has been presented that there was a gradual increase of ^{14}C-oleate incorporation into lipids when 1 to 5 mM of 2,3-diphosphoglycerate was added to the incubation mixture. A change in the distribution of radioactivity under the above conditions was also observed. At low concentrations of this compound, stimulation of incorporation of

Table 8

Incorporation of ^{14}C-oleate Into Lipids by Washed Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	192,000	100	166,000	87	12,200	6	13,500	7
Pellet + 3 μm 2,3-DPGA	213,000	100	185,000	87	13,500	14	14,100	7
Pellet + 3 μm α-GP	183,400	100	113,000	62	25,900	14	44,700	24
Pellet + 3 μm α-GP + 5 μm Ni^{++}	183,200	100	109,000	60	30,300	16	43,800	24
Pellet + 3 μm α-GP + 1 μm 2,3-DPGA	203,000	100	141,000	69	28,300	14	33,800	17
Pellet + 3 μm α-GP + 5 μm 2,3-DPGA	263,000	100	134,000	51	24,000	9	105,100	40
Pellet + 3 μm α-GP + .05% cutscum	238,000	100	117,000	49	19,400	8	101,700	43
Pellet + 3 μm α-GP + .25% cutscum	222,000	100	121,000	54	25,500	12	75,700	34
Pellet + 3 μm α-GP + 0.1 μm CTP	199,000	100	135,000	68	25,900	13	38,300	19
Pellet + 3 μm α-GP + 10 μm EDTA	251,000	100	139,000	55	23,000	9	89,700	36

Conditions: same as Table 5. Pellet washed 1x with 0.05M Tris buffer pH 7.55 containing 10^{-3}M DTT

labeled substrate into TG was observed with a concomitant decrease of labeling in polar lipids. When the concentrations of 2,3-DPGA was increased, however, a gradual shift of incorporation from TG to polar lipids was evident. These observations might suggest that this compound, at low concentrations, stimulated the activity of PA-phosphohydrolase and DG-acyltransferase, at higher concentrations, however, an inhibitory effect was observed. At all of the concentrations tested, meanwhile, the activity of the α-GP-acyltransferases was increased.

It may also be seen that there was a difference in the total amount of lipid synthesized as well as in the distribution of radioactivity in the various lipids when cutscum, CTP, or EDTA was included in the incubation mixture. The effects of CTP and EDTA were further investigated.

Results in Table 9 show that CTP up to a concentration of 1 mM enhanced the incorporation of ^{14}C-oleate into lipids. All of the increased label incorporated was recovered in the polar lipid fractions while the amount of labeled fatty acid incorporated into neutral lipids was not altered. At higher concentrations of CTP (5 and 10 mM), however, a total decrease in incorporation was observed.

The observation that the incorporation of ^{14}C-oleate into lipids by the pellet fraction of M. smegmatis Cell-Free Extract was stimulated by the addition of EDTA to the incubation mixture led to a further study of the effects of this

Table 9

Effects of CTP on the Incorporation of ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet + 2.5 μm α-GP	181,300	100	80,700	45	27,000	15	73,600	40
Pellet + 2.5 μm α-GP + 0.1 μm CTP	236,000	100	82,200	35	27,700	12	126,100	53
Pellet + 2.5 μm α-GP + 1.0 μm CTP	230,700	100	77,100	33	24,300	11	129,300	56
Pellet + 2.5 μm α-GP + 5.0 μm CTP	152,000	100	63,000	42	20,600	14	68,400	45
Pellet + 2.5 μm α-GP + 10 μm CTP	132,900	100	57,500	43	19,400	15	56,000	42

Conditions: 3.8 mg washed 30,000 xg/1 hr. pellet, oleic acid (sp. act. 10 μCi/μmole) 441,000 dpm, phosphate buffer pH 7.55 100 μmoles, 0.1 μmole CoA, 5 μmoles ATP, 5 μmoles Mg^{2+}, 5 μmoles mercaptoethanol, in a total volume of 1 ml. 1 hr. incubation at 35 C

compound. Results shown in Tables 10 and 11 demonstrate that when EDTA or KF was included in the incubation system where Tris·HCl buffer was used a change in the percent distribution of radioactivity among the various lipids occurred compared to parallel studies where potassium phosphate buffer was used. It is thus evident that the changes observed when EDTA was added were not due to the effect of this compound per se, but to that of the Na^+ or K^+ ions.

Evidence was presented earlier that the incorporation of labeled fatty acid into TG was enhanced by glycolytic products. Results in Table 11 indicate that the degree of stimulation of incorporation by glyceraldehyde 3-phosphate and dihydroxyacetone phosphate was similar, however, a much greater stimulation effect was seen with 2,3-diphosphoglycerate. The greater stimulatory effect of the latter compound suggests that possibly the mode of action of these glycolytic intermediates might be due to an indirect effect, by generation of ATP, rather than by some direct action of these compounds upon the various enzymes.

4. Effect of Triose Phosphates on ^{14}C-oleate Incorporation into Lipids by Supernatant and Pellet Fractions of Old M. Smegmatis Cell-Free Extract. Cell-Free Extracts were prepared from late logarithmic phase glucose and glycerol cells. The effects of certain triose phosphates on lipid synthesis were studied. Results are presented in Tables 12 and 13.

Table 10

Effects of EDTA on the Incorporation of ^{14}C-oleate into Lipids by Washed Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Tris + 2.5 µm α-GP	173,000	100	23,700	14	16,100	9	133,100	77
Tris + 2,5 µm α-GP + 11 µm EDTA + 15 µm Mg^{2+}	202,000	100	52,200	26	58,900	29	91,300	45
Tris + 5 µm KF + 2.5 µm α-GP	229,000	100	38,500	17	39,000	17	151,900	66
Tris + 5 µm KF + 2.5 µm α-GP + 11 µm EDTA + 15 µm Mg^{2+}	228,000	100	65,000	29	68,700	30	93,900	41
PO_4 + 5 µm KF + 2.5 µm α-GP	207,000	100	59,600	29	45,500	22	102,400	49
PO_4 + 5 µm KF + 2.5 µm α-GP + 11 µm EDTA + 15 µm Mg^{2+}	255,000	100	78,500	31	62,900	25	113,300	44

Conditions: 300 µl washed 30,000 xg/1 hr. pellet, resuspended in 0.05M Tris buffer, pH 7.5 with 10^{-4}M DTT, oleic acid (sp. act. 10 µCi/µmole) 488,000 dpm, 0.1 µmole CoA, 5 µmoles ATP, 5 µmoles Mg^{2+}, 5 µmoles mercaptoethanol, buffer (1M phosphate pH 7.5 N 1M Tris pH 7.5) 100 umoles in a total volume of 1 ml.1 hr. incubation at 35 C

Table 11

Effects of Tris Buffer and Phosphate Buffer ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Tris + 2.5 μm GA3P	135,000	100	96,800	72	9,700	7	27,900	21
Tris + 2.5 μm DHAP	149,000	100	108,000	72	9,900	5	31,200	21
Tris + 2.5 μm 2,3-DPGA	182,000	100	149,000	82	9,300	5	23,400	13
Tris + 2.5 μm GA3P + 5 μm KF	183,000	100	140,000	77	12,500	7	30,200	16
Tris + 2.5 μm DHAP + 5 μm KF	188,000	100	143,000	76	12,100	7	32,300	17
Tris + 2.5 μm 2,3-DPGA + 5 μm KF	206,000	100	172,000	84	11,900	6	22,000	10
PO_4 + 2.5 μm GA3P + 5 μm KF	190,000	100	159,000	83	14,100	7	17,800	10
PO_4 + 2.5 μm DHAP + 5 μm KF	196,000	100	167,000	85	12,300	6	17,000	8
PO_4 + 2.5 μm 2,3-DPGA + 5 μm KF	233,000	100	207,000	89	10,600	5	15,800	6

Conditions: same as Table 10

Table 12

Incorporation of ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glucose Grown Old* M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	136,000	100	113,500	83	9,900	7	13,000	10
Pellet + 5 μm GA3P	128,000	100	103,700	81	10,200	8	13,900	11
Pellet + 5 μm α-GP	226,000	100	66,800	30	30,000	13	128,800	57
Supernatant	216,000	100	160,000	74	6,900	3	49,000	23
Supernatant + 5 μm α-GP	242,000	100	129,000	53	19,100	8	93,700	39

Conditions: same as Table 2

* late Log-phase cells

Table 13

Incorporation of ^{14}C-oleate into Lipids by Pellet Fractions Prepared From Glycerol Grown Old* M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
Pellet	113,000	100	74,800	66	14,800	13	23,900	21
Pellet + 5 μm GA3P	94,000	100	59,600	63	15,200	16	19,600	21
Pellet + 5 μm GA3P + 5 μm NaF	87,000	100	51,400	59	16,700	19	18,800	22
Pellet + 5 μm DHAP	102,000	100	66,000	65	14,400	14	21,400	21
Pellet + 5 μm α-GP	143,000	100	35,500	25	25,800	18	81,800	57
Supernatant	202,000	100	104,000	52	31,200	15	66,700	33
Supernatant + 5 μm α-GP	240,000	100	109,000	45	44,000	18	87,900	37

Conditions: same as Table 2 except that 5 μmoles NaF was omitted

* late Log-phase cells

In these experiments it was shown that no appreciable difference in the distribution of labeling was found to occur with α-glycerophosphate when Cell-Free Extracts from either old or young cells was used. On the other hand, contrary to the case found with young cells, the various glycolytic products were inactive with Cell-Free Extracts from older cells.

It is unlikely that the ineffectiveness of the glycolytic products in the old cell preparations was due to a change of enzyme profile in these fractions. It is possible that the inability of these added compounds to stimulate lipid synthesis was due to a large intracellular pool of these substrates already present in the cells as a result of a lowered metabolic rate in the older cells.

III. Glycolytic Products as Possible Direct Acyl Acceptors for Triglycerides Synthesis.

In mammalian systems it has been shown that dihydroxyacetone phosphate may act as an acyl group acceptor. This alternate pathway may eventually terminate in TG biosynthesis. In this study experiments were performed to determine if dihydroxyacetone phosphate and some of the other glycolytic products, which showed stimulatory activity, acted as acyl group acceptors.

Experiments with labeled glucose (Table 14), Fructose

Table 14

Incorporation of ^{3}H-Glucose* into Lipids by Total Cell-Free Extracts of Young Glycerol Grown M. Smegmatis

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
^{3}H-1-glucose	1992	100	471	24	366	18	1155	58
^{3}H-1-glucose + 5 μm F^-	1587	100	435	27	270	17	882	56
^{3}H-6-glucose	1506	100	468	31	297	20	741	49
^{3}H-6-glucose + 5 μm F^-	2157	100	2130	29	402	19	1125	52

Conditions: 400 μl total cell-free extract, 100 μmoles oleate in 5% BSA, phosphate buffer, pH 7.55 100 μmoles, 0.05 μmoles CoA, 5 μmoles ATP, 5 μmoles Mg^{2+}, 5 μmoles mercaptoethanol in a total volume of 1 ml. 1 hr. incubation at 35C

* 2.5 μCi ^{3}H-glucose added

1,6-diphosphate (Table 15), and 3-phosphoglycerate (Table 16 and 17) showed no appreciable incorporation of radioactivity into neutral lipids, phosphatidic acid, or phospholipids. These experiments show the inability of glycolytic products to serve as direct substrates in lipid synthesis.

IV. Incorporation of ^{14}C-glycerophosphate into Lipids by Supernatant and Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts.

When using ^{14}C-oleic acid as a tracer in lipid biosynthesis studies, some anomalous conclusions may be drawn because several mechanisms may be occurring simultaneously.

1. de novo synthesis by direct acylation of α-glycerophosphate
2. acylation of an endogenous acceptor lipid such as diglyceride or its derivatives
3. exchange of fatty acids by endogenous lipids

In an attempt to eliminate or reduce the other possible interferring factors in studying de novo lipid synthesis, ^{14}C-glycerophosphate and ^{14}C-glycerol were used as markers in the following experiments.

A comparison of the abilities of supernatant, pellet, and ammonium sulfate precipitated supernatant protein

Table 15

Incorporation of ^{14}C-Fructose 1,6-diphosphate into Lipids by Pellet Fractions Prepared From Young Glucose Grown M. Smegmatis Cell-Free Extracts

	Triglyceride	Diglyceride	Polar Lipids
	cpm	cpm	cpm
Fructose 1,6-diphosphate (^{14}C*)	52	326	1388
Fructose 1,6-diphosphate (^{14}C*) + isomerase	56	618	1486

Conditions: 300 µl washed pellet, 100 µmoles oleate in 5% BSA, phosphate buffer pH 7.55 100 µmoles, Mg^{2+} 5 µmoles, NaF 5 µmoles, ATP 5 µmoles, CoA, .1 µmoles mercaptoethanol 5 µmoles, 2 units of aldolase, 20 units of isomerase, in a total volume of 1 ml, incubation at 35C for 1 hr.

* 0.5 µCi ^{14}C-Fructose 1,6-diphosphate

Table 16

Incorporation of ^{14}C-3-phosphoglycerate into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

		Triglyceride	Diglyceride	Polar Lipids
		cpm	cpm	cpm
3-phosphoglycerate (^{14}C)	0 time	30	42	189
3-phosphoglycerate (^{14}C)	30 minutes	76	110	312
3-phosphoglycerate (^{14}C)	60 minutes	48	72	458

Conditions: 300 µl washed 30,000 xg/1 hr. pellet, 0.5 µCi 3PGA, 5 µmoles Mg^{2+} and 5 µmoles mercaptoethanol, 30 µmoles oleoylCoA, 100 µmoles phosphate buffer in a total volume of 1 ml, incubation at 35C

^{14}C-3PGA (sp. act. 43 µCi/µmole)

Table 17

Incorporation of ^{14}C-3-phosphoglycerate into Lipids by Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	cpm	%	cpm	%	cpm	%
3PGA ^{14}C (0.5μCi) + 2.5 μm 3PGA-(^{12}C) + 100 nm oleate	1,800	100	904	51	250	14	382	22
^{14}C-oleate + 80 nm ^{12}C-oleate + 2.5 μm 3PGA(^{12}C)	245,000	100	230,000	94	9,600	4	5,600	2
^{14}C-oleate + 80 nm ^{12}C-oleate	228,600	100	214,000	94	9,400	4	4,800	2

Conditions: phosphate buffer pH 7.55 100 μmoles, CoA 0.1 μmole, 5 μmoles each of ATP, Mg^{2+}, NaF, and mercaptoethanol, 300 μl washed 30,000 xg/1 hr. pellet in a total volume of 1 ml, 1 hr. incubation at 35C

when added, 488,000 dpm oleate (sp. act. 10 μCi/μmole) was used.

fractions of glycerol and glucose grown M. smegmatis Cell-Free Extracts to incorporate ^{14}C-α-GP into lipids is shown in Table 18. Radioactivity in lipids was recovered by the glass filter paper method (see Materials and Methods).

These results indicate that pellet fractions prepared from Cell-Free Extract of glucose grown M. smegmatis cells was more efficient in incorporating ^{14}C-α-GP into lipids than pellet fractions from glycerol grown cells. However, when the abilities of supernatant Cell-Free Extract fractions from glucose and glycerol cells to incorporate the labeled substrate were compared, no significant difference was detected. Such an observation is consistent with that found when ^{14}C-oleate was used as a marker (Tables 12 and 13).

The effect of the pH of the incubation mixture on the incorporation of ^{14}C-α-GP into lipids was also studied. It may be seen that when compared to pH 7.5, the radioactivity incorporated into lipids by pellet fractions at pH 8.6 was lower while the label in lipids synthesized by supernatant fractions and by supernatant fractions plus pellet fractions was greatly increased. High pH slightly favors lipid formation by supernatant proteins precipitated with 0-20% saturation ammonium sulfate.

The mechanisms involved in the observed pH effect are

Table 18

Incorporation of ^{14}C- α-glycerophosphate into Lipids by Supernatant, Pellet, and Ammonium Sulfate Precipitated Fractions Prepared From M. Smegmatis* Cell-Free Extracts

	pH of incubation mixture		nm GP incorp/mg Protein/hr.
Glycerol grown	7.5	Supernatant	22.0
		Pellet	0.7-0.8
		Supernatant & Pellet	7-8
	8.6	Supernatant	56.0
		Pellet	0.2-04
Glucose grown	7.5	Supernatant	23.1
		Pellet	2.0
		Supernatant & Pellet	11-12
		0-20% AMS	8.1
		20-40% AMS	6.6
	8.6	Supernatant	41.0
		Pellet	0.4-0.5
		Supernatant & Pellet	25-26
		0-20% AMS	9.3

Conditions: Protein 30 µl (500-1000µg), α-mercaptoethanol 3.6 µmoles, Mg^{2+} 0.45 µmoles, 1M Tris buffer pH 7.5 25 µl, oleoylCoA 3.0 nmoles, ^{14}C-α-glycerophosphate 540 nmoles, 337,000 dpm in a total volume of 170 µl. 1 hr. incubation at 30C. Lipids recovered by GFP method

* mid-late logarithmic phase cells used

not known. It may be that the differences in response to pH by the supernatant and pellet fractions could be due to a difference in the relative amounts of the various enzymes distributed in these two fractions.

It is possible that the activity of α-GP-acyltransferases was higher at pH 7.5, and the lower radioactivity recovered in lipids in the supernatant fraction at this pH was not a result of anabolism but of catabolism. The higher activity of phosphatidate phosphohydrolase at pH 7.5 could result in the production of larger amounts of neutral lipids which might be more susceptible to the action of lipases. Consequently, the increased reactivity of the α-GP-acyltransferases would be masked by the increased action of the lipolytic enzymes.

The decreased amount of α-GP incorporated per milligram of protein in the pellet fraction when compared to the supernatant fraction of the Cell-Free Extract was undoubtedly due to the presence of a large amount of structural proteins in the pellet fraction.

Experiments reported here (Table 19) and performed by Barakat (11) and Walker (unpublished experiments) have determined that Mg^{2+}, oleoyl-CoA are required for supernatant Cell-Free Extract fractions to catalyze the incorporation of ^{14}C-α-GP into lipid. Neutral lipid synthesis is enhanced by the addition of higher concentrations of Mg^{2+}.

Table 19

Effect of Magnesium and OleoylCoA on the Incorporation of ^{14}C-glycerophosphate into Lipids by the Supernatant Fractions Prepared From Glucose Grown *M. Smegmatis* Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	dpm	%	dpm	%	dpm	%	dpm	%
Supernatant	23,800	100	5,980	25	10,800	45	6,980	29
Supernatant + 7.5 µm Mg^{2+}	22,800	100	9,600	42	8,400	37	4,790	21
Supernatant + 7.5 µm Mg^{2+} + 25 µm oleoylCoA	22,500	100	11,000	49	6,900	31	4,590	20

Conditions: 16 mg 30,000 xg/1 hr. supernatant, 0.05M Tris buffer pH 7.5 containing 10^{-3}M DTT, ^{14}C-glycerophosphate, 540 µmoles, 337,000 dpm, 2.5 µmoles Mg^{2+}, 5 µmoles oleoylCoA (1 hr. incubation at 30C) in a total volume of 1 ml

Increasing the concentration of oleoyl-CoA stimulates the formation of TG somewhat, however, the ratio of neutral lipids to polar lipids remained unchanged.

From these experiments it would appear that a Mg^{2+} activated phosphatidic acid phospholydrolase is present in the supernatant fraction. It would also seem that in this system oleoyl-CoA was acting as a limiting factor in the acylation of the diglyceride produced to form TG. A similar Mg^{2+} dependent PA- phosphohydrolase also appears to be present in the pellet fractions (Table 20).

The following sections detail experiments which were performed to optimize the conditions for lipid biosynthesis in cell free systems. In these experiments the effects of Mg^{2+}, ATP, CTP and other small molecules were studied.

V. Optimizing the Conditions for Lipid Synthesis

Early logarithmic phase glucose grown M. smegmatis cells were harvested, washed with 0.05 M Tris buffer pH 7.5 and resuspended in 0.05 M Tris buffer pH 7.5 containing 10^{-3}M DTT. Cell-Free Extracts were prepared and fractionated into supernatant and pellet fractions as covered in Materials and Methods. In the control experiments, 3.4 mg of supernatant and 2.5 mg of pellet, respectively, were incubated at 35C for 1 hour with 1.5 µm of α-glycerophosphate (1 µmole coldαGP + 5.6 µmoles ^{14}C-α-GP,

Table 20

Effect of Magnesium on the Incorporation of ^{14}C-α-GP into Lipids by Pellet Fraction Prepared From M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	dpm	%	dpm	%	dpm	%	dpm	%
Pellet	1,639	100	89	5	169	10	1,381	84
Pellet + 5 μmoles Mg^{2+}	1,938	100	293	15	522	27	1,123	58

Conditions: 2-4 mg washed 30,000 xg/1 hr. pellet protein suspended in 0.05M Tris buffer, pH 7.5 with 10^{-3}M DTT, ^{14}C-α-GP 1.03 μmoles, 672,000 dpm, potassium phosphate 100 μmoles, 5 μmoles KF, 100 μmoles oleoylCoA, 3 mg bovine albumin serum in a total volume of 1 ml, 1 hr. incubation at 35C

336,000 dpm), 0.1 μmole of oleoylCoA, 100 μmoles of phosphate buffer, pH 7.55, 5 μmoles each of KF and $Mgcl_2$, and 3 mg of BSA in a total volume of 1 ml. The amount of ^{14}C-α-GP incorporated into lipids was studied by varying the concentrations of Mg^{2+} (Table 21) and of BSA (Table 22) by omitting the KF, and by the addition of 5 μmoles of mercaptoethanol as well as various concentrations of KCl (Tables 23 and 24). The optimal condition for the incorporation of ^{14}C-α-GP into lipids by the supernatant and pellet fractions of glucose grown young M. smegmatis Cell-Free Extracts was found to be 1 mM DL-α-GP, 0.1 mM oleoylCoA, 100 mM phosphate buffer pH 7.55, 5 mM Mg^{2+} and 0.1% bovine serum albumin. The addition of 5 mM mercaptoethanol or 5mM KF or KCl resulted in inhibitions of biosynthesis.

The relationship between the enzyme concentration and the ^{14}C-α-GP incorporation was also studied. Results are shown in Figures 2 and 3. It may be seen that a linear relationship between protein concentration and ^{14}C-α-GP incorporation by supernatant fractions was observed only in the lower range of protein concentrations. At higher concentrations the incorporation of labeled substrate tended to level off. It is possible that with a large amount of enzymes, certain co-factor (s) would become rate limiting. The linear increase of radioactivity at the higher protein concentrations with the pellet fraction was probably due

Table 21

Effect of Varying Magnesium Concentrations on the Incorporation of ^{14}C-α-GP into Lipids by the Supernatant and Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	dpm	%	dpm	%	dpm	%	dpm	%
Supernatant + 1 μm Mg^{2+}	5,105	100	2,549	50	1,022	20	1,534	30
Supernatant + 3 μm Mg^{2+}	5,428	100	2,918	54	1,010	19	1,500	27
Supernatant + 5 μm Mg^{2+}	6,497	100	3,334	51	954	15	2,209	34
Supernatant + 10 μm Mg^{2+}	4,832	100	2,727	56	766	16	1,339	28
Pellet + 1 μm Mg^{2+}	3,890	100	609	16	521	13	2,760	71
Pellet + 3 μm Mg^{2+}	4,078	100	876	22	575	14	2,627	64
Pellet + 5 μm Mg^{2+}	4,592	100	936	20	609	13	3,047	67
Pellet + 10 μm Mg^{2+}	3,967	100	884	22	526	13	2,557	65

Conditions: see text

Table 22

Effect of Varying BSA Concentrations on the Incorporation of ^{14}C-α-GP into Lipids by the Supernatant and Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	dpm	%	dpm	%	dpm	%	dpm	%
Supernatant + 0.9 mg BSA	6,644	100	3,274	49	1,591	24	1,779	27
Supernatant + 1.5 mg BSA	5,089	100	2,505	49	1,064	21	1,520	30
Supernatant + 3.0 mg BSA	6,497	100	3,334	51	954	15	2,209	34
Supernatant + 6.0 mg BSA	3,742	100	1,224	33	1,823	49	695	18
Pellet + 0.9 mg BSA	5,812	100	1,226	21	554	10	4,032	69
Pellet + 1.5 mg BSA	4,971	100	1,117	23	660	13	3,194	64
Pellet + 3.0 mg BSA	4,592	100	936	20	609	13	3,047	67
Pellet + 6.0 mg BSA	4,570	100	451	10	399	9	3,720	81

Conditions: see text

Table 23

Effect of Mercaptoethanol, KF, and KCl on the Incorporation of ^{14}C-α-GP into Lipids by Supernatant Fractions Prepared From M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	dpm	%	dpm	%	dpm	%	dpm	%
Supernatant	6,497	100	3,334	51	954	15	2,209	34
Supernatant + 5 µm merc. ETOH	5,939	100	2,791	47	1,080	18	2,068	35
Supernatant + 5 µm KF	5,307	100	2,844	54	1,025	19	1,438	27
Supernatant + 25 µm KCl	4,845	100	2,760	57	962	20	1,123	23
Supernatant + 50 µm KCl	5,069	100	2,781	55	1,042	20	1,246	25
Supernatant + 100 µm KCl	4,856	100	2,621	54	1,088	22	1,147	24

Conditions: see text

Table 24

Effect of Mercaptoethanol, KF, and KCl on the Incorporation of ^{14}C-α-GP into Lipids by the Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	dpm	%	dpm	%	dpm	%	dpm	%
Pellet	4,592	100	936	20	609	13	3,047	67
Pellet + 5 µm merc. ETOH	4,194	100	892	21	551	13	2,751	66
Pellet + 5 µm KF	8,122	100	876	11	689	9	6,557	80
Pellet + 25 µm KCl	4,260	100	1,028	24	554	13	2,678	63
Pellet + 50 µm KCl	2,859	100	835	29	487	17	1,537	54
Pellet + 100 µm KCl	3,537	100	769	22	480	14	2,288	64

Conditions: see text

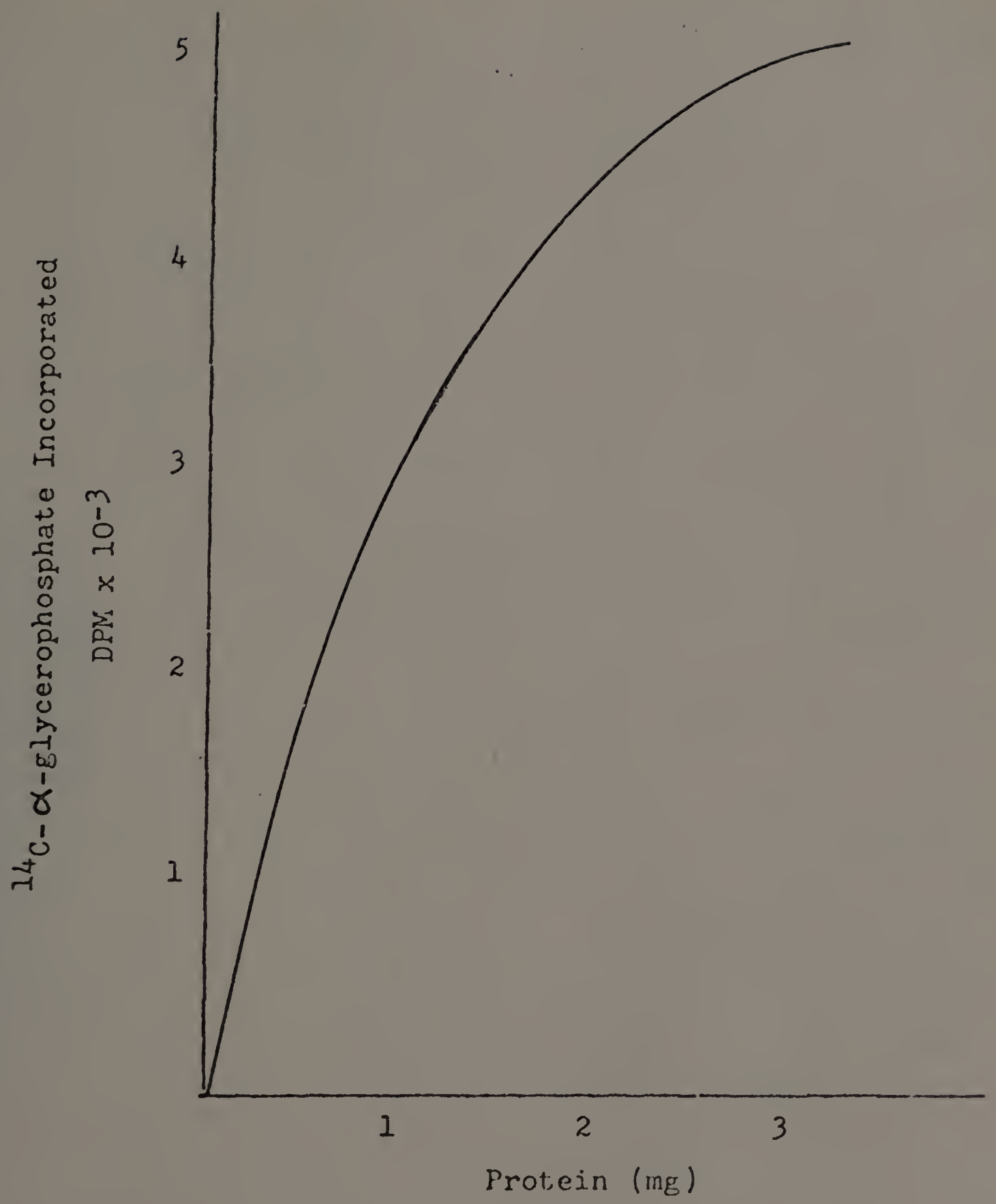

Figure 2

Relationship Between Protein Concentrations and ^{14}C-α-GP Incorporated by 30,000 xg/1 hr. Supernatant Fraction Prepared From M. Smegmatis Cell-Free Extracts.

Conditions: 0.5-3 mg supernatant proteins, 100 umoles pot. phosphate buffer at pH 7.55, 1 umoles DL -GP, 0.52 umole ^{14}C- -GP (336,000 dpm) 0.1 umole oleoylCoA, 1 mg BSA, 5 umoles Mg^{2-}, in a final volume of 1 ml 1 hr. incubation at 35C.

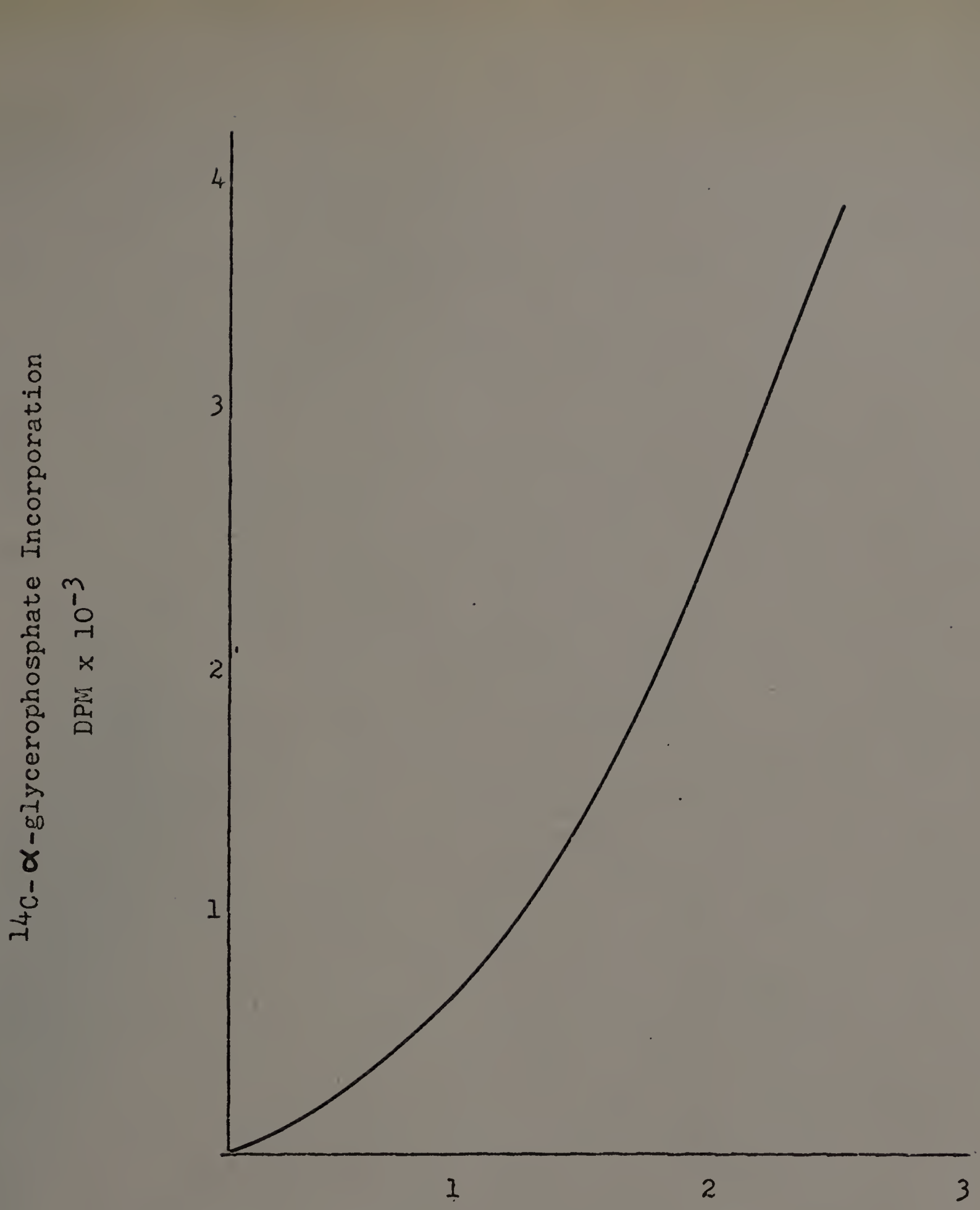

Figure 3

Relationship Between Protein Concentrations and ^{14}C-α-GP Incorporation by 30,000 xg/1 hr. Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts.

Same conditions as for Figure 2. 0.3-2.5 mg pellet protein used.

to the presence of a large portion of structural proteins in this fraction.

Figures 4 and 5 show a time-study of the incorporation of ^{14}C-α-GP by supernatant and pellet fractions respectively. It may be seen that under the conditions used with the supernatant fraction, that incorporation was linear up to 15 minutes. In the case of the pellet fraction, linearity occurred up to 60 minutes. These experiments also show that a considerable portion of the label is incorporated into neutral lipid by the supernatant fraction. In the case of the pellet fraction most of the labeling occurred in the polar lipids. Whether such a variation is due to the difference in the PA-phosphohydrolase activities can not be determined. However, the higher TG/DG ratio observed in the supernatant fraction may indicate that the DG-acyltransferase activity is higher in this fraction.

1. Effect of Magnesium on the Incorporation of ^{14}C-α-GP into Lipids by the Supernatant Fraction Prepared from M. Smegmatis Cell-Free Extracts. Various concentrations of $MgCl_2$, ranging from 0 to 5mM, and other co-factors found to be optimal for ^{14}C-α-GP incorporation were incubated with the supernatant fraction of glucose grown young M. smegmatis Cell-Free Extracts. Results are summarized in Figure 6. It clearly indicates that there is a phosphatidic acid phosphohydrolase present in the cytosol

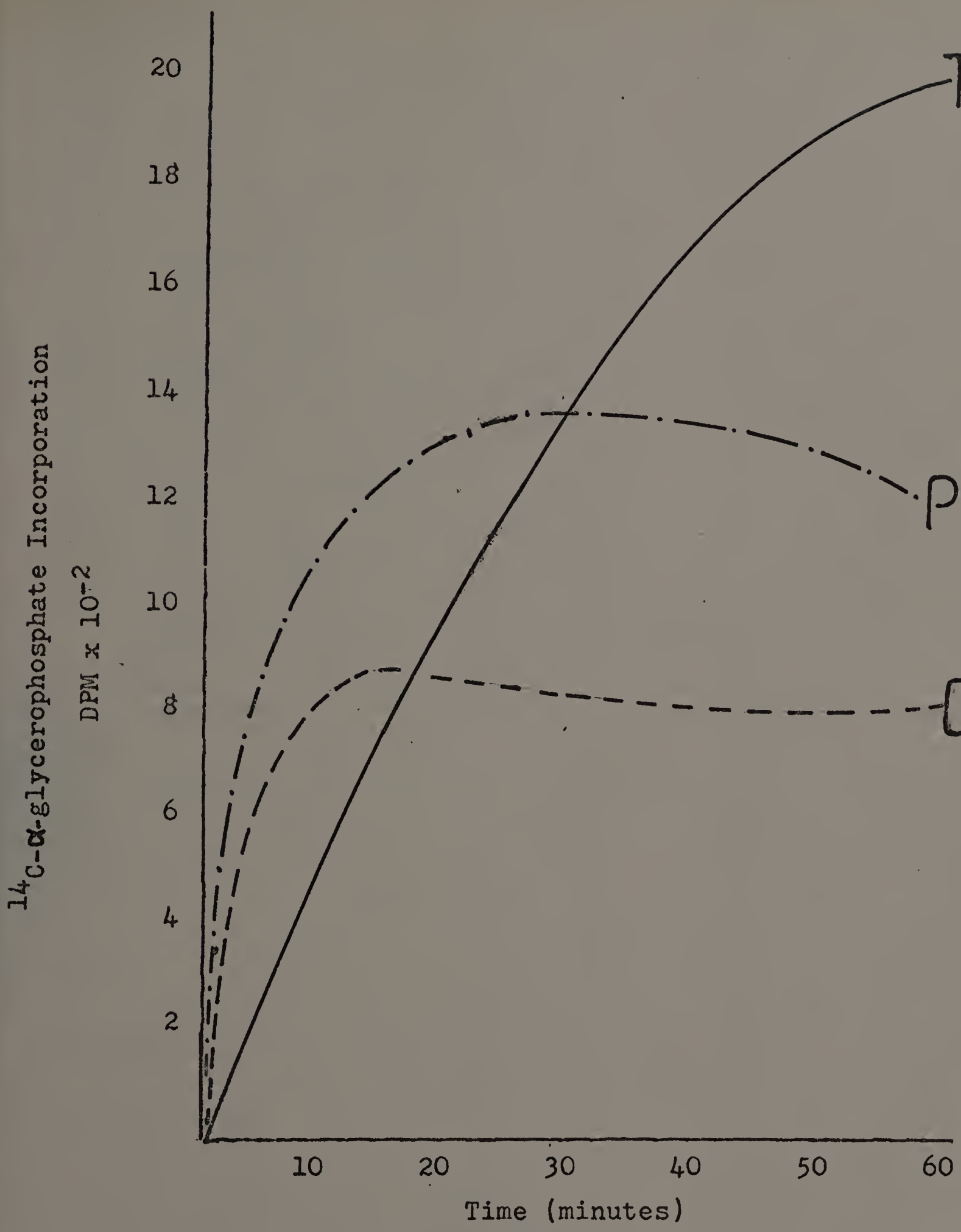

Figure 4

Relationship Between the Incorporation of ^{14}C-α-GP into Lipids and Incubation Time.

Conditions: 2-3 mg 30,000 xg/1 hr. supernatant protein, other co-factors same as for Figure 2.

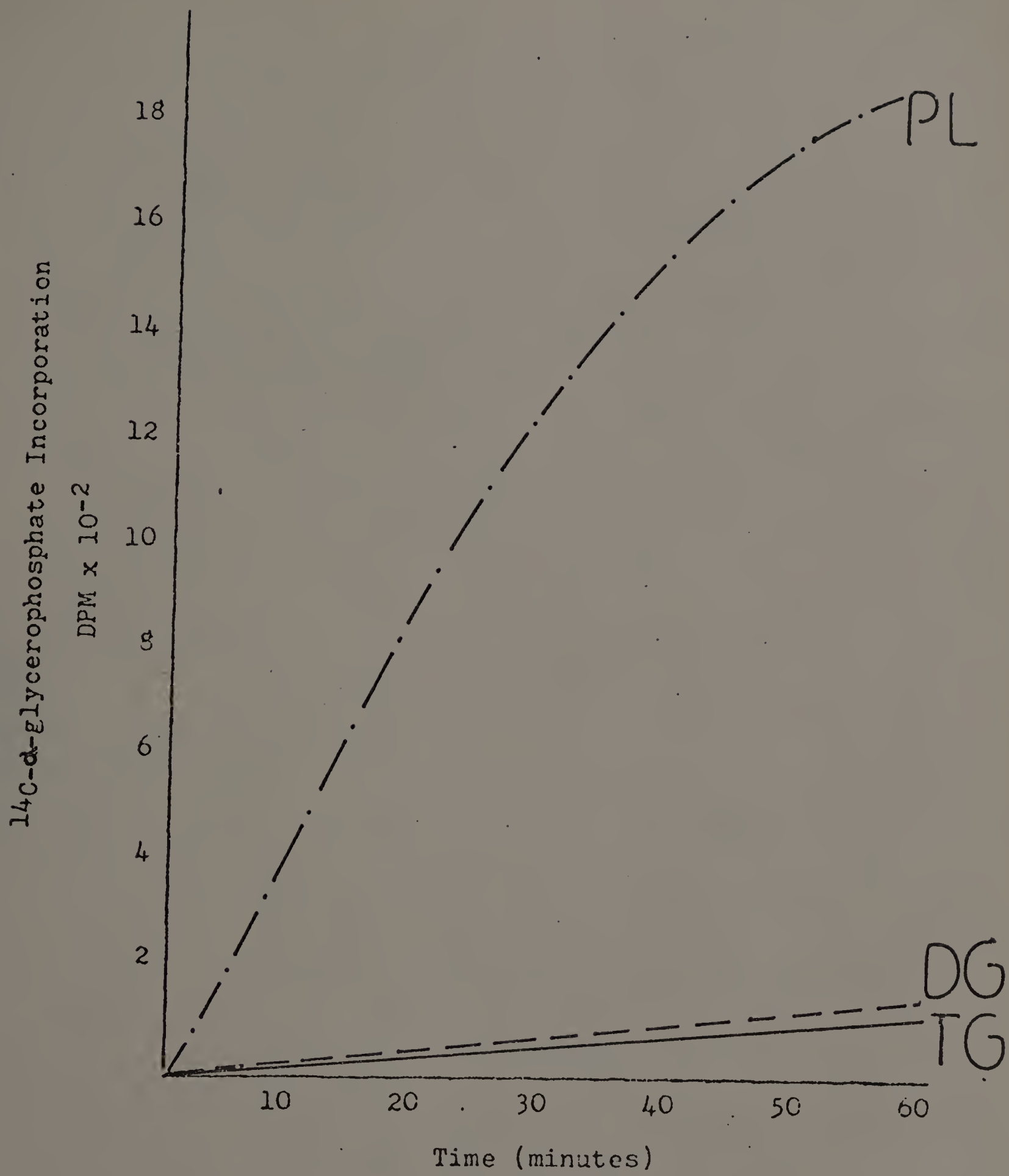

Figure 5

Relationship Between the Incorporation of ^{14}C- α-glycerophosphate into Lipids and Incubation Time.

Conditions: 2-2.5 mg 30,000 xg/1 hr. pellet protein, other co-factors same as for Figure 2.

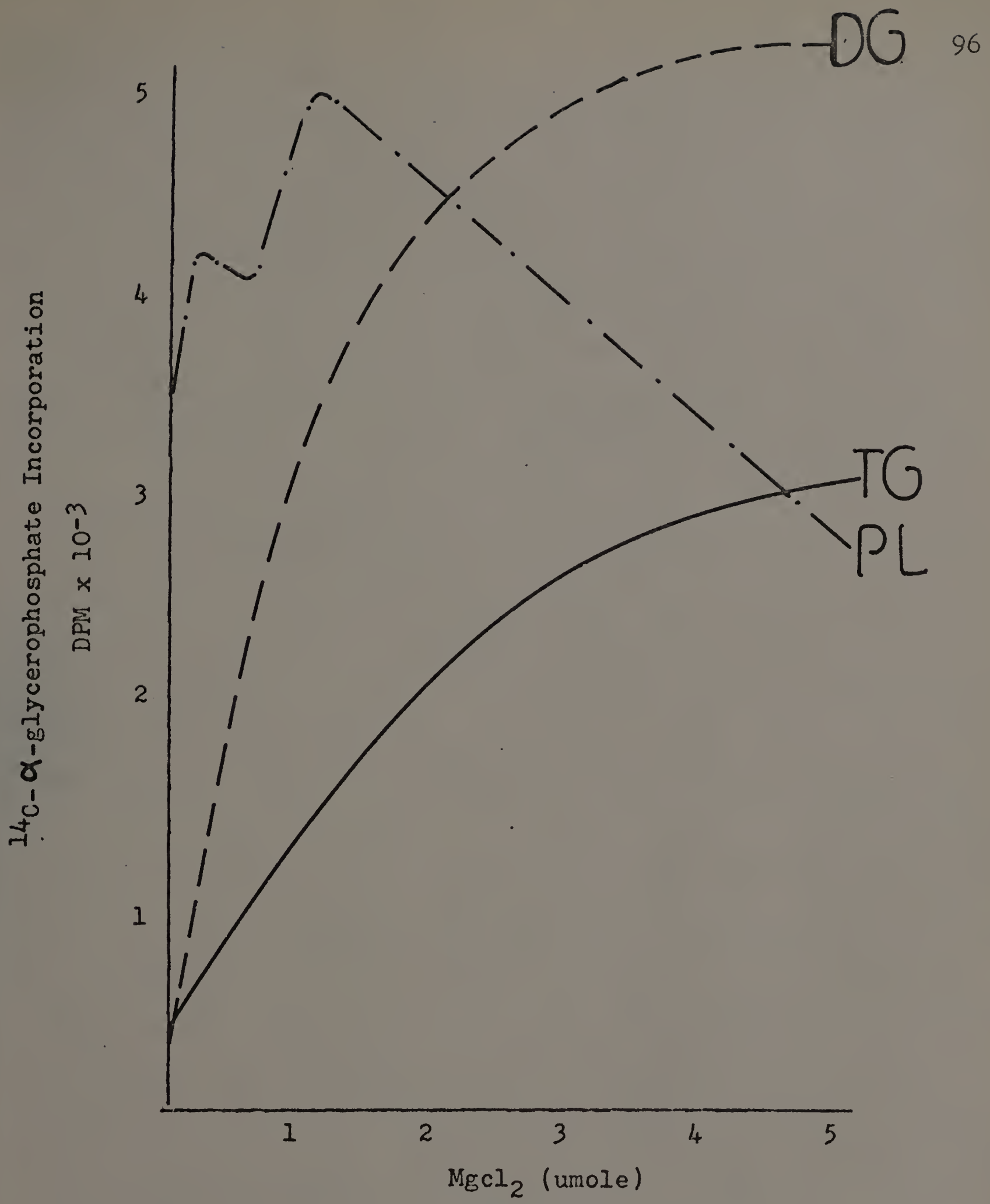

Figure 6

Effect of Mg^{2-}Concentration on the Incorporation of ^{14}C-α-GP into Lipids by Supernatant Fraction Prepared from M. Smegmatis Cell-Free Extracts. 1.5 mg supernatant proteins with varying concentration of Mg^{2-}, 15 minutes incubations.

Other conditions same as for Figure 2.

fraction and that its activity is stimulated by magnesium ions. It may also be seen that low concentration of Mg^{2+} (up to 1 mM) also activated α-GP acyltransferases. At higher concentrations the Mg^{2+} was inhibitory. These results are consistent with observations presented earlier in this study (Table 19).

2. Effects of ATP and CTP on the Incorporation of ^{14}C- α-Glycerophosphate into Lipids by Supernatant and Pellet Fractions of M. Smegmatis Cell-Free Extracts. The enhancement of lipid biosynthesis by ATP in mammalian systems has been reported by Johnston et. al. (72), by Smith and Hubscher (139), and by Smith and co-workers (140). ATP and CTP was also observed in this study to stimulate lipid formation (Figures 7, 8, 9 and 10).

Smith and Hubscher (139) and Johnston and associates (72) have suggested that CoA may be recycled in the presence of ATP (and indirectly CTP) to form acyl-CoAs from endogenous fatty acids which are better substrates in the acylation reactions than is the added acylCoA. The existence of specific acyl-transferases in mycobacteria was suggested by the observation that fatty acids are very specifically distributed in the neutral lipids and phospholipids of these orgnaisms. However, this fact is disputed by the earlier studies of this investigation where labeled fatty acids were used. If ATP and CTP indeed

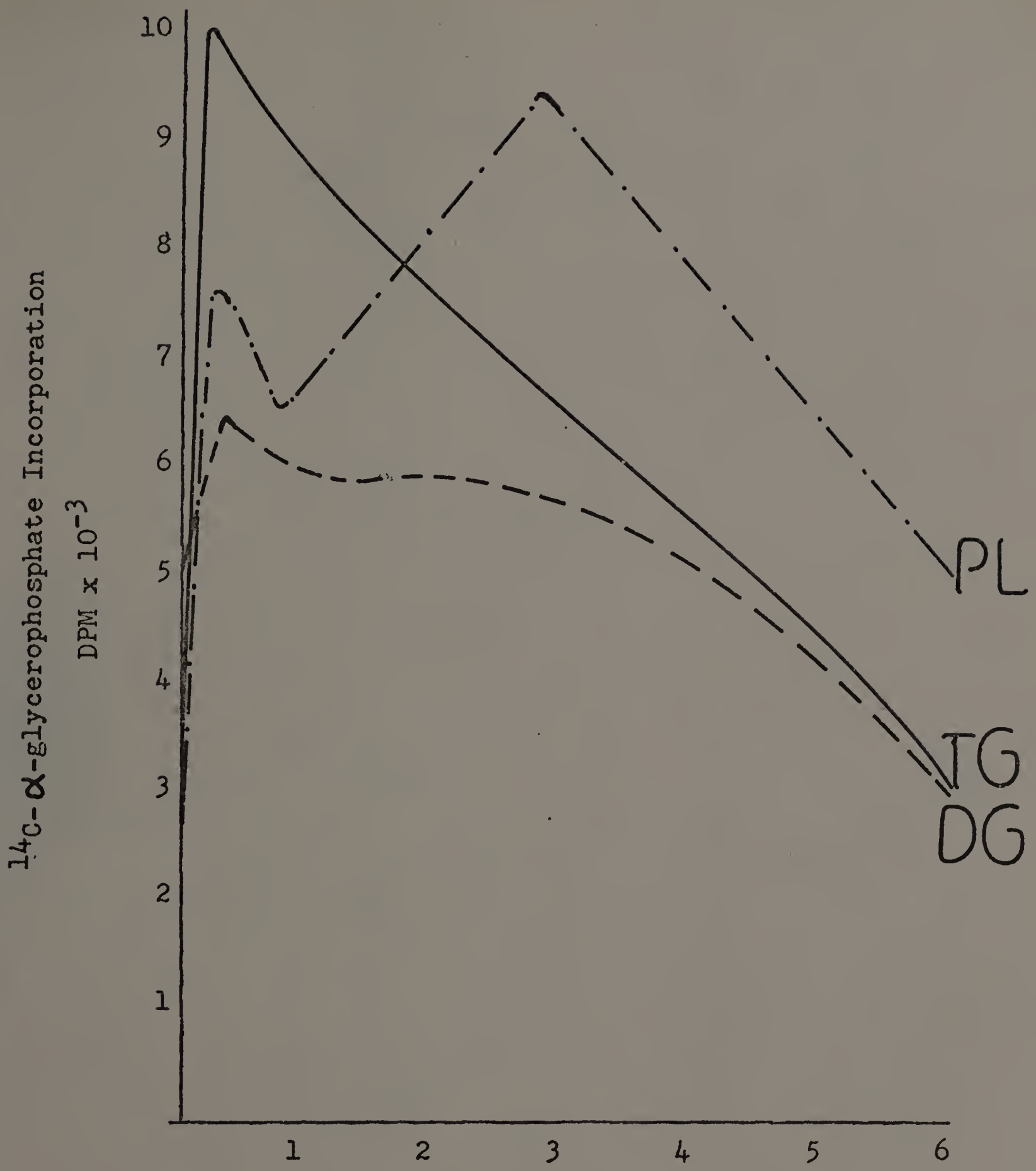

Figure 7

Effect of ATP on Incorporation of ^{14}C-α-GP into Lipids by 30,000 xg Supernatant Fraction of M. Smegmatis Cell-Free Extracts.

1.5 mg supernatant protein, 15 minutes incubation at 35C. Other co-factors same as for Figure 2.

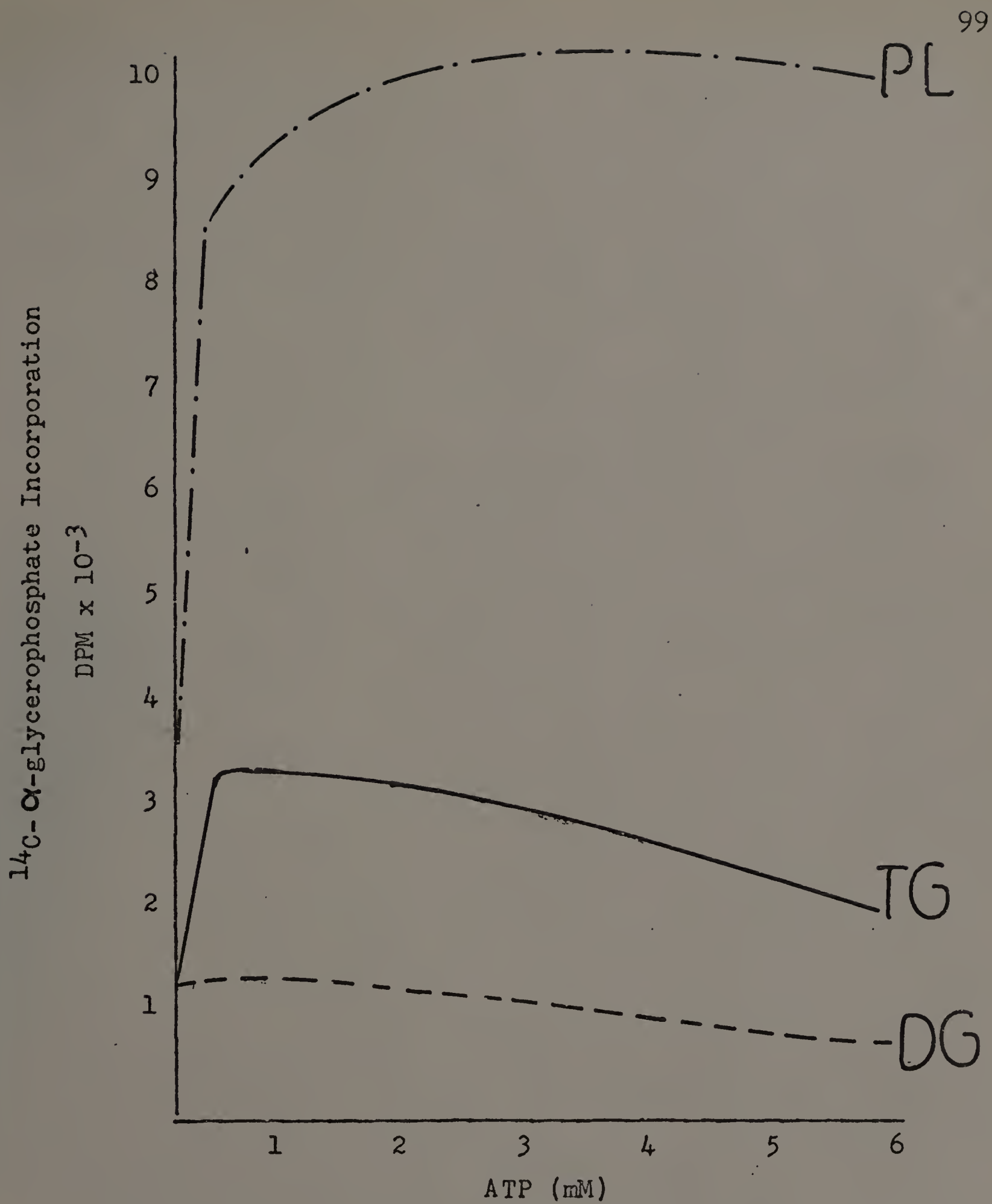

Figure 8

Effect of ATP on the Incorporation of ^{14}C-α-GP into Lipids by 30,000 xg Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts.

2.5 mg of pellet protein. Other conditions same as for Figure 2.

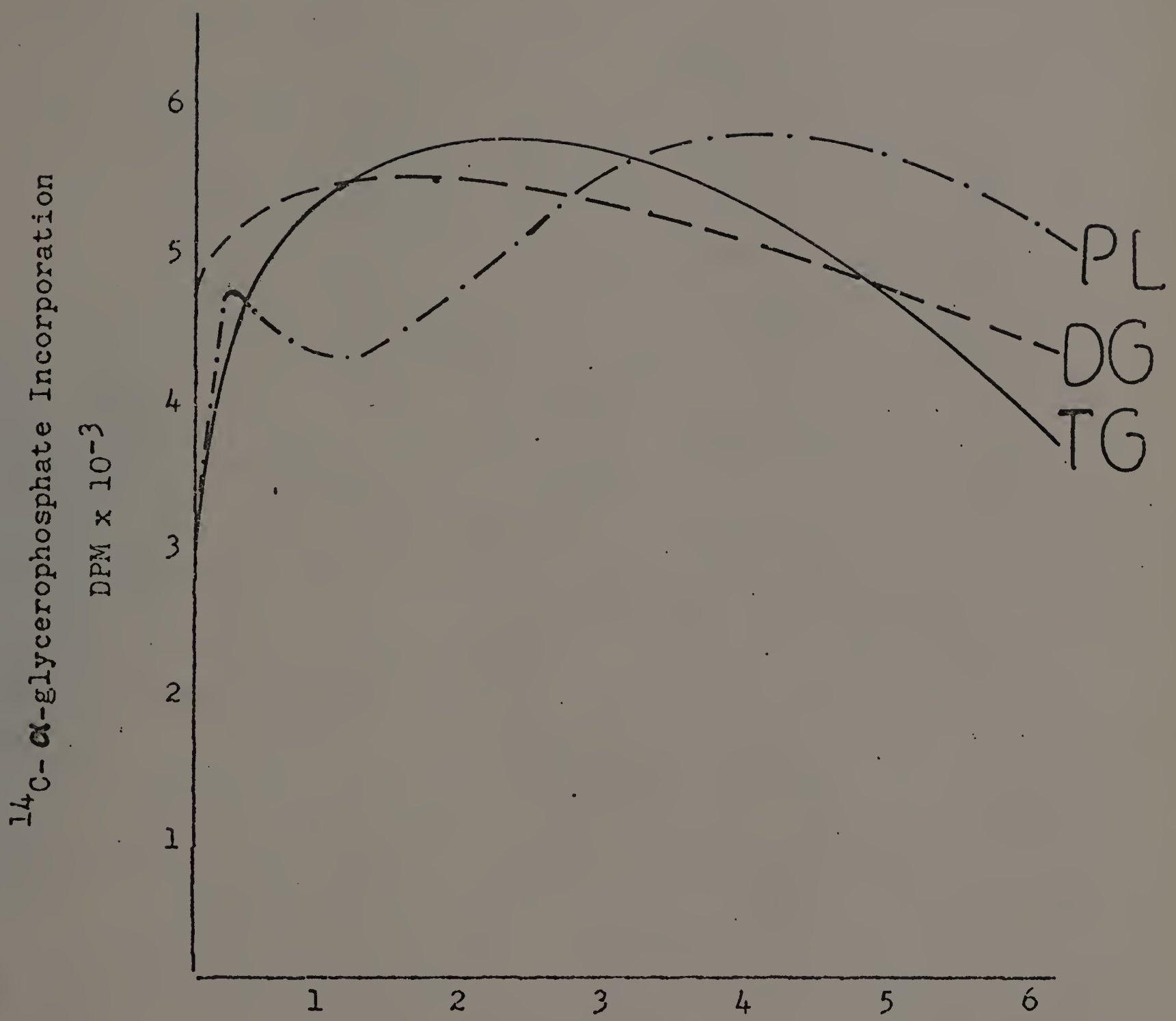

Figure 9

Effect of CTP on the Incorporation of ^{14}C-α-glycerophosphate into Lipids by 30,000 xg Supernatant Fractions Prepared From M. Smegmatis Cell-Free Extracts.

Conditions same as for Figure 8.

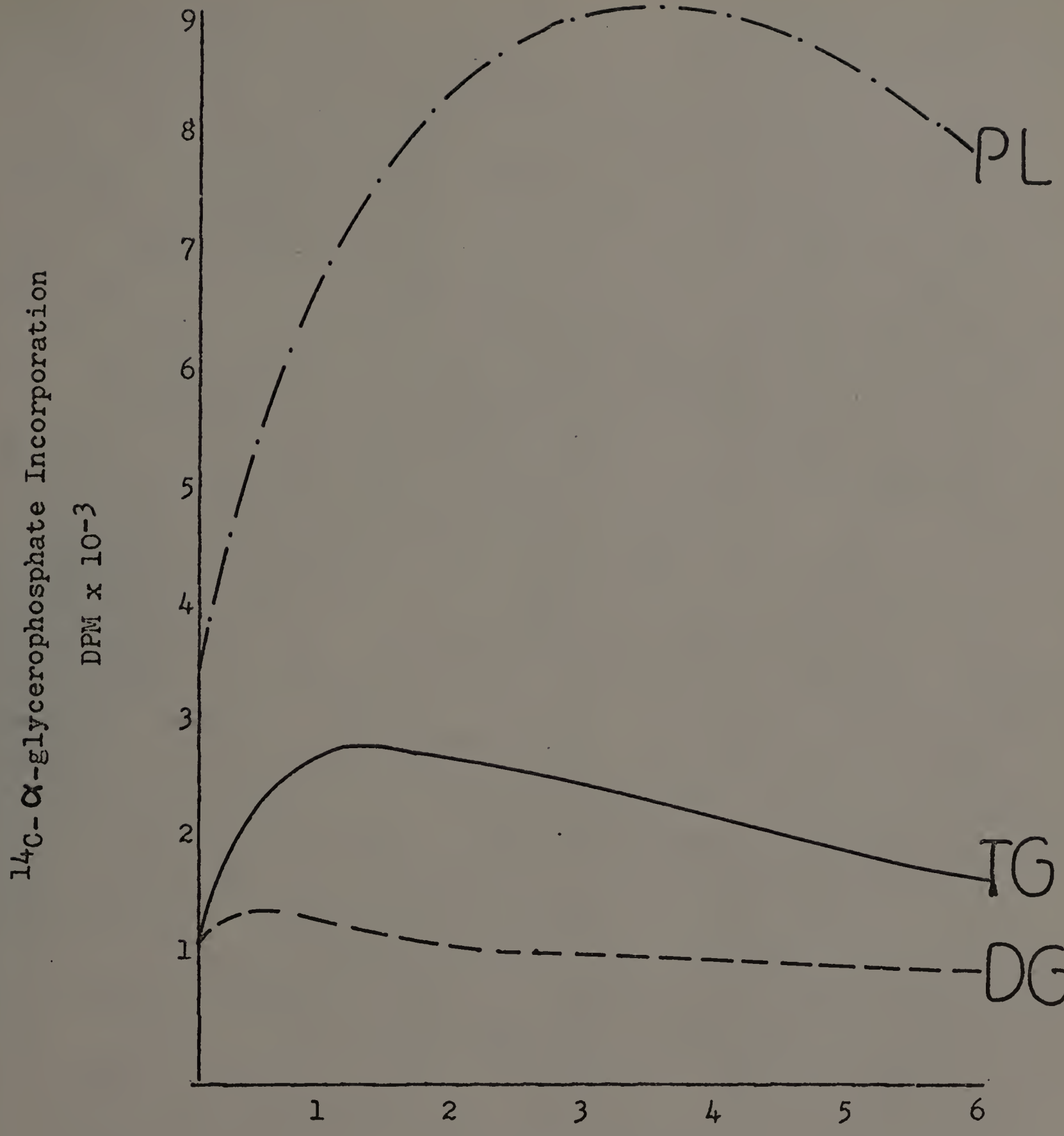

Figure 10

Effect of CTP on the Incorporation of ^{14}C-α-glycerophosphate into Lipids by Washed 30,000 xg Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts.

Conditions same as for Figure 9.

facilitates the formation of acylCoAs from endogenous fatty acids which would then be preferentially utilized by the acyl-transferases in acylating α-glycerophosphate, then the amount of ^{14}C- fatty acid incorporated into lipids should be reduced. Results in Table 9 indicate that the incorporation of labeled fatty acids is stimulated by the addition of small amounts of CTP.

The similarity in the stimulation of lipid synthesis at ATP and CTP (Figures 7 and 9, and 8 and 10) tends to suggest that their mode of action is the same. Comparing the results summarized in Figure 5 with those in Figures 7 and 10, and those in Figure 6 with Figures 8 and 9, it is evident that the action of the DG-acyltransferase enzyme (s) was greatly enhanced by the nucleotides. Data also indicate that the activities of α-glycerophosphate acyltransferases and that of phosphatidic acid phosphohydrolase were also stimulated. However, whether these nucleotides activated these enzymes by increasing the V_{max}, or lowering the K_m or some other mode of action awaits further investigation.

3. Effect of KF on ^{14}C- α-Glycerophosphate Incorporation into Lipids. The effect of F^- ion on ^{14}C- α-GP incorporation into lipids was also studied. Results are presented in Table 25. It may be seen that the incorporation of labeled substrate is slightly inhibited only at a very

Table 25

Effect of KF on the Incorporation of the ^{14}C- -GP into Lipids by Supernatant and Pellet Fractions Prepared From Glucose Grown Young M. Smegmatis Cell-Free Extracts

	Total Incorporation		Triglyceride		Diglyceride		Polar Lipids	
	cpm	%	dpm	%	dpm	%	dpm	%
Supernatant	10,911	100	3,020	28	4,981	45	2,910	27
Supernatant + 0.3 μm KF	9,171	100	2,891	31	4,551	50	1,729	19
Supernatant + 1.0 μm KF	10,537	100	2,685	26	4,565	43	3,287	31
Supernatant + 10 μm KF	10,706	100	3,293	31	5,208	48	2,205	21
Supernatant + 50 μm KF	9,317	100	1,424	15	4,315	46	3,578	38
Pellet	5,593	100	1,077	19	1,135	20	3,381	61
Pellet + 0.3 μm KF	4,738	100	1,030	21	1,166	25	2,542	54
Pellet + 1.0 μm KF	5,143	100	1,094	21	1,364	27	2,685	52
Pellet + 10.0 μm KF	5,040	100	1,070	21	1,170	23	2,800	56
Pellet + 50.0 μm KF	4,157	100	606	15	896	21	2,655	64

Conditions: 2.6 mg or 1.5 mg of washed 30,000 xg pellet and supernatant protein, respectively, 100 μmoles potassium phosphate buffer $_{p}H$ 7.55, 1 μmoles DL-α-GP, 0.52 μmoles ^{14}C-α-GP (336,000 dpm), 0.1 μmoles CoA, 1 mg bovine serum albumin, final volume 1 ml, incubation 1 hr. at 35C

high concentration of F^-. The activity of the PA-phosphohydrolase, which was low in the pellet and high in the supernatant fraction was unaffected by F^- at 10 mM. However, high concentrations of this ion inhibited the enzyme equally well in both the pellet and supernatant fractions.

VI. Phosphatidate Phosphohydrolase.

It is generally believed that there are two phosphatidate phosphohydrolases present in mammalian tissues, one is membrane-bound while the other is a soluble enzyme. The respective preferences of these enzymes for either soluble or membrane-bound substrate has been discussed in the literature review. The magnesium dependency or independency and differences in biological function has also been reviewed.

In these studies preliminary results suggested that the PA-phosphohydrolase from M. smegmatis was a single enzyme. In order to probe this hypothesis further several experiments were perfromed to study the properties of this enzyme and to determine if in fact only one PA-phosphohydrolase is present in M. smegmatis. The following sections detail those experiments.

1. Cytosol PA-Phosphohydrolase. Its Activity Against Soluble Substrates. Previous studies have presented evidence indicating that ^{14}C-α-glycerophosphate could be

successfully incorporated into TG, DG, and polar lipids by both supernatant and pellet fractions of M. smegmatis Cell-Free Extract. In the following experiments supernatant proteins of this organism were precipitated with ammonium sulfate by a procedure similar to that which was used satisfactorily to partially purifying the enzyme PA-phosphohydrolase in mammalian cell-free extracts (136, 137, 139). Fractions thus obtained were tested for their ability to incorporate ^{14}C-glycerol and ^{14}C-α-glycerophosphate into neutral and polar lipids. Results are summarized in Table 26.

It may be seen that by the simple expedient of making the supernatant fraction to 20% and to 40% saturation with ammonium sulfate resulted in nearly all of the enzymes involved in the de novo lipid synthesis from glycerol being rendered sedimentable causing a two to three fold purification. Though very little difference in specific activity was observed between the 0-20% and 20-40% saturation AMS precipitated proteins, it appears from these data that higher activities of phosphatidate phosphohydrolase and DG-acyltransferase were found in the 0-20% fraction while the 20-40% fraction appears to contain more α-GP-acyltransferase activity. Supporting results for such an observation were obtained from experiments in which Tris buffer, supernatant proteins, and 20-40% and 40-60% AMS

Table 26

Incorporation of ^{14}C-α-glycerophosphate and ^{14}C-glycerol by Total Cell-Free Extract, Pellet, Supernatant and Various Ammonium Sulfate Precipitated Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts

		DPM Per Mg Protein			
		Total Incorporation	Triglycerides	Diglycerides	Polar Lipids
^{14}C-α-GP incorporation	Total cell-free extract	787	39	148	331
	Supernatant	782	33	98	361
	0-20% AMS	1,765	150	311	747
	20-40% AMS	2,189	141	316	994
	40-100% AMS	331	-	-	-
	Pellet	99	-	-	-
^{14}C-glycerol incorporation	Total cell-free extract	14,480	1,067	3,065	4,486
	Supernatant	20,047	366	2,386	8,101
	0-20% AMS	42,799	6,509	6,499	10,361
	20-40% AMS	76,689	3,816	6,784	23,116
	Pellet	4,249	21	187	2,412

Conditions: 400 µl protein, 5 µmoles Mg^{2+}, 50 n moles oleoylCoA, ^{14}C-α-glycerophosphate 540 n moles, 336,000 dpm or ^{14}C-glycerol (sp. act. 16 µCi/µmole) 1,257,800 dpm in a total volume of 1 ml. 1 hr. incubation at 30C. 5 µmoles ATP added when ^{14}C-glycerol was used

fractions, respectively, were added to lipids pre-synthesized from ^{14}C- α-glycerophosphate by 0-20% AMS proteins (Table 27).

Repeated freezing and thawing has been used to partially purify the enzyme phosphatidate phosphohydrolase obtained from mammalian tissues. In order to determine what effect such a procedure would have on the activities of PA-phosphohydrolase, α-glycerophosphate acyltransferases, and DG-acyltransferase of M. smegmatis, the 0-20% and 20-40% AMS fractions were subjected to a process of freezing and thawing five times in a period of five weeks. The ^{14}C-glycerol incorporation into various lipids by these two fractions was again studied (Table 28 and Figure 11). It was demonstrated that the DG-acyl-transferases was nearly completely inactivated by such a treatment while the PA-phosphohydrolase was not, or was only slightly affected.

Because of the high nucleoprotein content of the Cell-Free Extracts it was felt that these compounds could have been responsible for the poor separation of enzymatic activities in the 0-20% and 20-40% AMS fractions. Fresh Cell-Free Extract was then treated first with manganous sulfate according to the method of Kaufman (78). After removal of the nucleoproteins by centrifugation, the supernatant fraction was then subjected to AMS fractionation. The

Table 27

Effect of Addition of Supernatant Fractions and Various Ammonium Sulfate Precipitated Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts on the Incorporation of ^{14}C-α-GP into Lipids by 0-20% AMS Fraction

Incubation Time		^{14}C- -glycerophosphate Incorporation (DPM)			
1 hr.	1/2 hr.	Total Lipids	Triglyceride	Diglyceride	Phosphatidic Acids
Control	Extract $\bar{W}$ C:M (2:1)	11,066	538	1,778	4,536
Control	+ 0.05M Tris buffer pH 7.5	10,372	969	2,925	3,919
Control	+ 2 mg Supernatant Protein	12,396	1,331	3,429	4,410
Control	+ 5 mg 20-40% AMS Protein	12,008	541	1,923	4,601
Control	+ 5 mg 40-60% AMS Protein	8,850	523	1,568	3,560

Conditions: control tube contains 3.5 mg 0-20% AMS protein, ^{14}C-α-GP 540 nmoles, 336,000 dpm 3.6 µmoles mercaptoethanol, 5 µmoles Mg^{2+}, 30 nmoles oleoylCoA, 0.05M Tris buffer, pH 8.6 in a final volume of 1 ml, 5 x above mixtures were incubated at 30C for 1 hr. 1/5 of the sample was removed to determine the distribution of radioactivity in various lipids. 1/4 of the remaining sample was distributed in each of 4 tubes with additions as indicated in the Table and incubated for an additional 1/2 hr. at the end of incubation, the distribution of radioactivity among the lipids was again determined.

Table 28

Effect of Repeated Freezings and Thawings on the Incorporation of ^{14}C-glycerol by 0-20% and 20-40% Saturation Ammonium Sulfate, Precipitated Supernatant Protein Prepared From M. Smegmatis Cell-Free Extracts

	DPM Per Mg of Protein			
	Total Incorporation	Triglyceride	Diglyceride	Polar Lipids
0-20% AMS	19,400	3,960	5,000	7,900
20-40% AMS	21,300	3,660	5,300	6,800

Conditions: same as Table 26

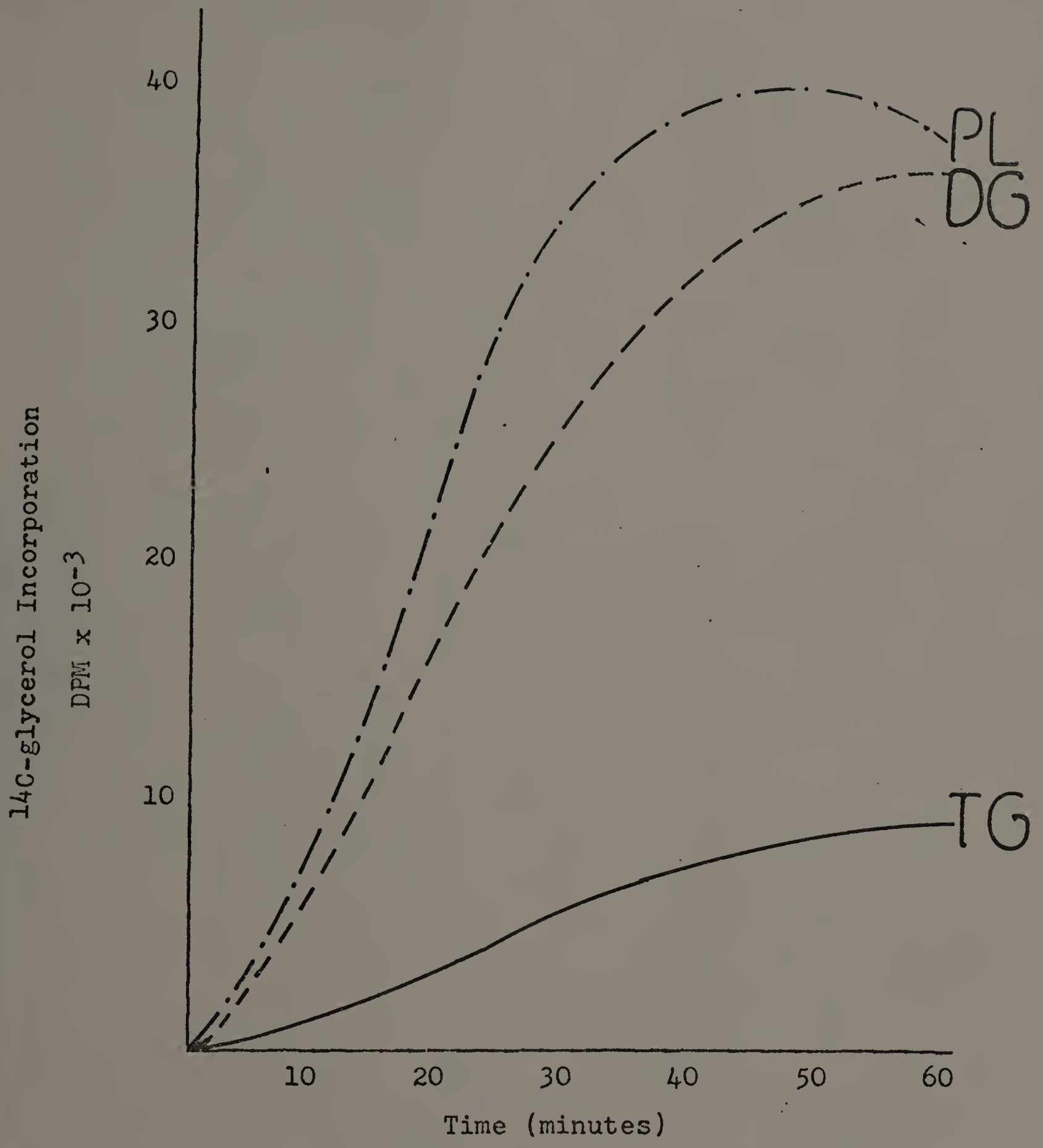

Figure 11

A Time Study of the Incorporation of ^{14}C-glycerol into Lipids by 0-20% AMS Precipitated Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts.

Conditions same as for Table 27.

incorporation of ^{14}C-glycerol, and the effects of KF and Tween-20 on its incorporation, into lipids by the AMS fractions were investigated. Data obtained from these studies (Tables 29 and 30) indicate that although the procedure was unsatisfactory for separating α-GP-acyltransferases from other lipid synthesizing enzymes, AMS frationation does appear to concentrate more of the PA-phosphohydrolase activity in the 20-40% AMS fraction.

Since the TG/DG ratio is similar in the 0-20% and 20-40% AMS fractions (Table 29), it was not clear whether the higher counts in TG in the latter fraction was due to a greater activity of DG-acyltransferase or to a larger amount of DG formed. This aspect was pursued later in this investigation.

Evidence has also been presented that Tween-20 inhibits lipid biosynthesis, probably at the level of the acylation of α-GP.

The effect of KF on various lipid synthesizing enzymes was variable. Inhibition of DG-acyltransferase activity was observed while a stimulatory effect on PA-phosphohydrolase was also noted. The paradoxical effect of KF on PA-phosphohydrolase activity as revealed in Table 30 and in Table 25 was probably due to the amount of K^+ presented in the incubation system. This enhancement effect upon neutral lipid formation by these ions was reported earlier

Table 29

Incorporation of ^{14}C-glycerol into Lipids by Ammonium Sulfate Salt Fractionated "Nucleoprotein-free" Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts

	DPM Per Mg of Protein				
	Total Incorporation	Triglyceride	Diglyceride	Phosphatidic Acids	Other Polar Lipids
0-20% AMS	35,400	5,630	8,180	19,700	1,560
20-40% AMS	35,100	8,440	12,790	11,900	1,540
20-40% AMS + 1% Tween-20	1,900	-	-	-	-

Conditions: 4-5 mg AMS fractionated supernatant protein, ^{14}C-glycerol (sp. act. 16μCi/μmole) 414,000 dpm, 5 μmoles Mg^{2+}, 60 nmoles oleoylCoA, 5 μmoles ATP, Tris buffer .05M, pH 7.5, in a final volume of 1 ml. 1 hr. incubation at 35C

Table 30

Effects of KF and Tween 20 on the Incorporation of ^{14}C-glycerol into Lipids by 0-20% and 20-40% AMS Fractionated "Nucleoprotein-free" Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts

Incubation Time		^{14}C-glycerol Incorporation (DPM)				
1 hr.	Additional 30 minutes	Total Lipids	Triglyceride	Diglyceride	Phosphatidic Acids	Other Polar Lipids
Control		67,900	6,420	7,960	37,040	13,470
Control	+ 20-40% AMS	65,450	7,820	9,340	31,020	14,250
Control	+ 20-40% AMS + 1% Tween 20	65,400	6,480	8,160	35,670	11,440
Control	+ 20-40% AMS + 20 µm KF	68,550	6,580	13,475	29,490	15,280

Conditions: control tube contained ^{14}C-glycerol (sp. act. 16µCi/µmole) 413,800 dpm, 5 µmoles Mg^{2+}, 60 nmoles oleoylCoA, 5 µmoles ATP, 1.5-3 mg 20-40% AMS supernatant protein, 0.05M Tris pH 8.5 in a final volume of 1 ml

4 x above mixtures were incubated at 30C for 1 hr. 1/4 was removed, lipids extracted and counted. 1/3 each of the remaining portion was distributed into each of 3 tubes with additions as indicated in the Table and incubated at 35C for an additional 30 minutes. At the end of the incubation, lipids were extracted and counted (procedures described in Materials and Methods)

(Tables 10 and 11). It is thus possible that high concentration of F^- ions indeed inhibit PA-phosphohydrolase activity, however, this inhibitory effect could be abolished or obscured by the greater stimulatory effect of the K^+ ions.

From the above studies, it is apparent that cytosol PA-phosphohydrolase can efficiently utilize soluble substrates. In order to determine its ability in hydrolyzing membrane-bound substrates, "membrane-bound" phosphatidic acids were synthesized and hydrolytic experiments were performed. Results of these studies are presented in the following sections.

2. Synthesizing "Membrane-Bound" Phosphatidic Acids. In the following experiments, 2 to 4 mg of 30,000 xg unwashed pellet fractions of M. smegmatis Cell-Free Extract suspended in 0.05 Tris buffer, pH 8.6 was incubated with ^{14}C-glycerol (sp. act. 16 µCi/µmole) 413,860 dpm, 5 µmoles of ATP, 60 µmoles of oleoylCoA, 5 µmoles of magnesium chloride, and 3.6 µmoles of mercaptoethanol in a total volume of 1 ml. The effects of various concentrations of NaF as well as the effect of incubation time on PA synthesis by the above system was studied. Results of these studies are shown in Figures 12 and 13, respectively.

Again it was seen that low concentrations of NaF caused an increase in labeling of triglycerides at the expense of

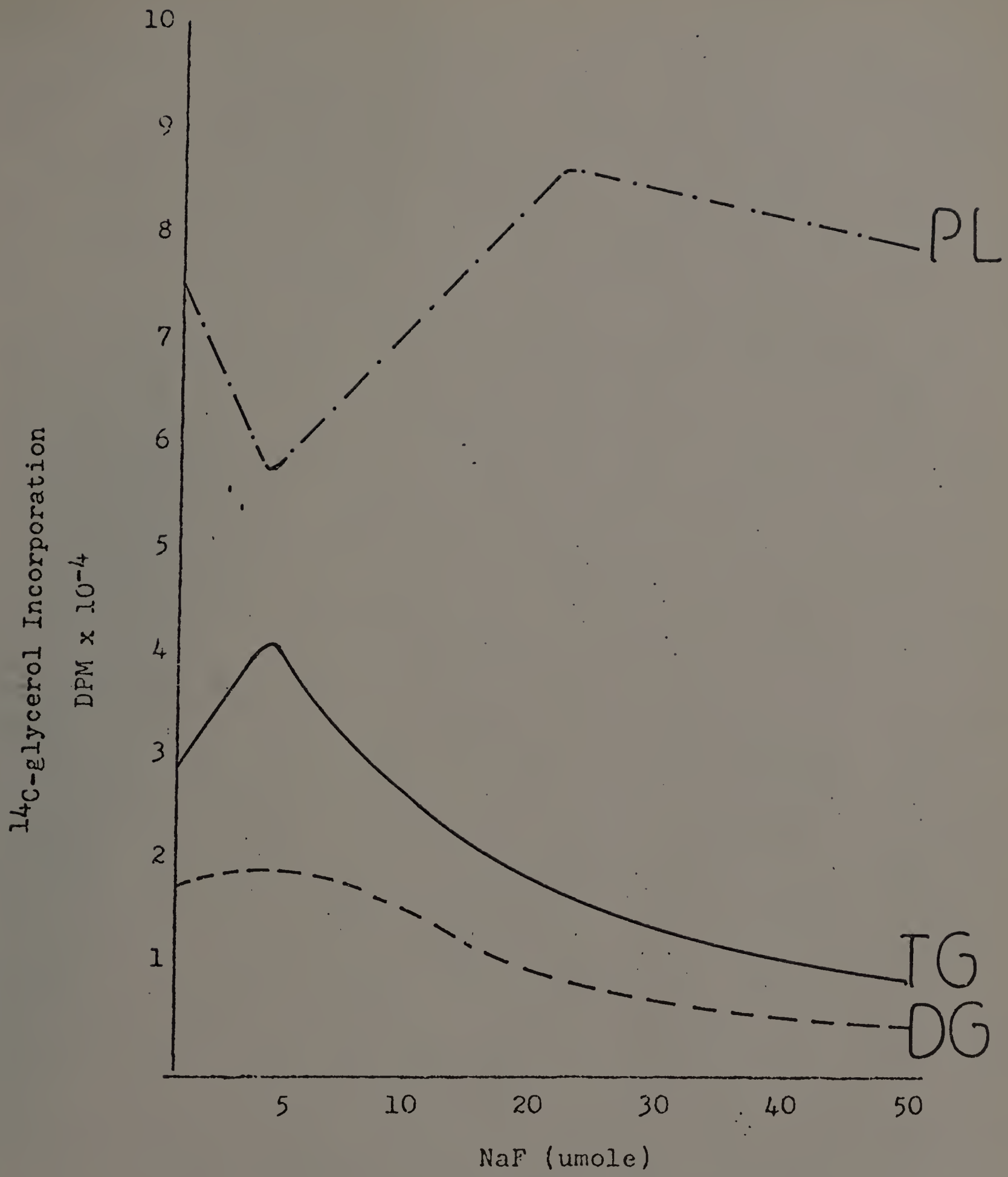

Figure 12

Effect of NaF on the Incorporation of ^{14}C-glycerol into Lipids by 30,000 xg Unwashed Pellet Prepared From M. Smegmatis Cell-Free Extracts.

Conditions: covered in text. 30 minutes incubation at 35C.

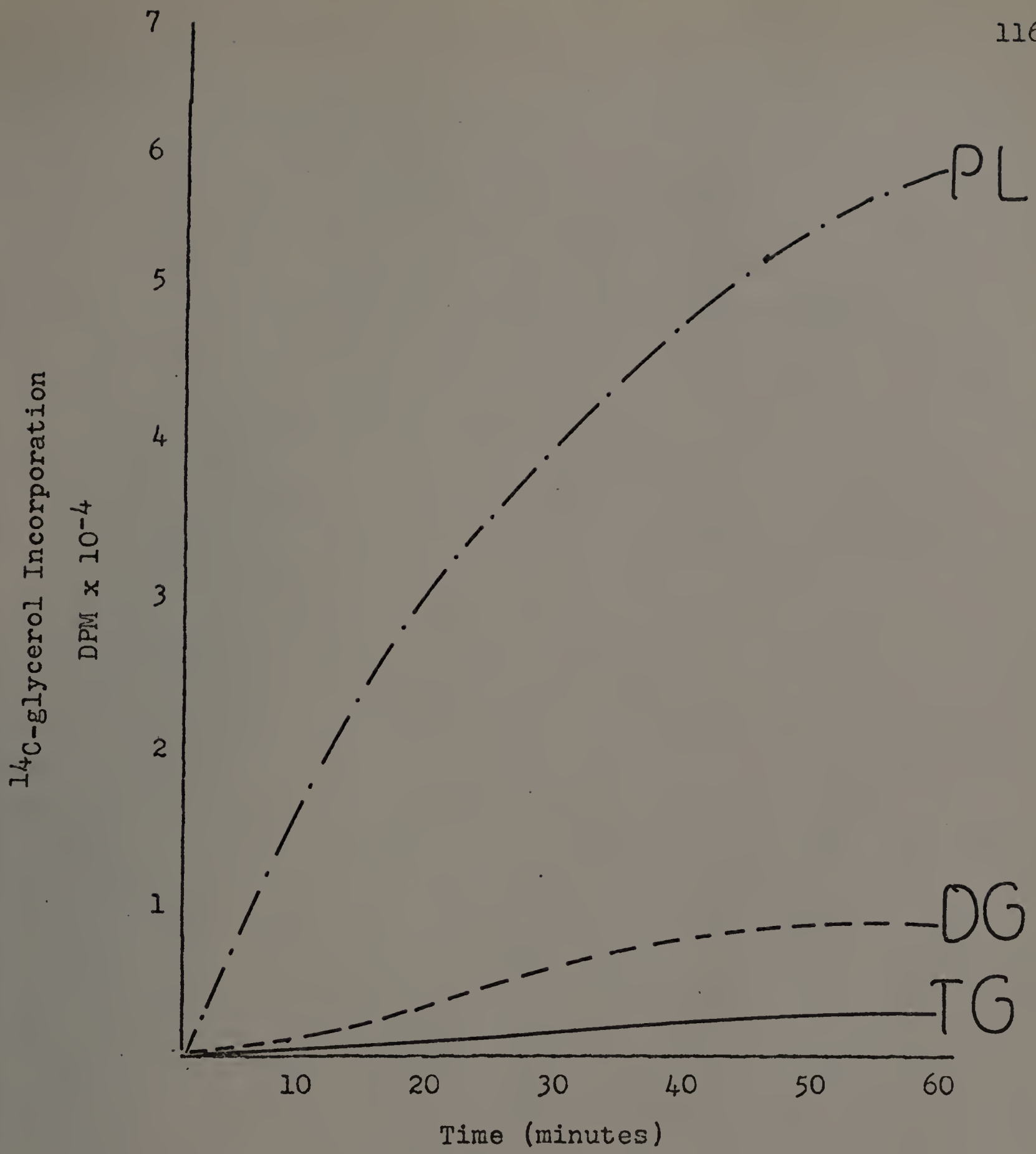

Figure 13

A Time Study of the Incorporation of ^{14}C-glycerol into Lipids by 30,000 xg Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts.

Conditions covered in text. 20mM NaF added.

labeling of the polar lipids. At the higher concentrations of fluoride ion, neutral lipid formation was inhibited, indicative of the inhibition of the PA-phosphohydrolase. It may also be seen from Figure 13 that a one hour incubation is suitable for the synthesis of "membrane-bound" phosphatidic acids.

The polar lipid fraction was analyzed by TLC and found to be composed of from 88-90% phosphatidic acid.

In order to obtain a substantial amount of membrane-bound substrate, the pellet proteins and other co-factors used in the foregoing experiments were scaled up 10-15 times and incubated at 35C for one hour. In an attempt to remove the unused ^{14}C-glycerol, the incubation mixture was filtered through a column of Sephadex G-25. The filtrate (referred to as "pre-synthesized membrane lipids") was used in the subsequent experiments.

3. Studies of the Effects of Phosphatidate Phosphohydrolase Against Membrane-Bound Substrate. To examine the activity of phosphatidic phosphohydrolase against membrane-bound substrate, 2.5 µmoles $Mgcl_2$ and 200 µl each of 0.05 M Tris buffer pH 7.5, pellet, supernatant, and 0-20%, 20-40%, and 40-60% AMS proteins of "nucleoprotein-free" supernatant fraction of M. smegmatis Cell-Free Extract, respectively, were distributed into each of six tubes. To each of these tubes, 200 µl of the filtrate containing

"pre-synthesized membrane lipids" was added and incubated at 35C for 30 minutes.

It had been found (Table 31) that the membrane-bound PA was hydrolyzed by both the pellet and supernatant PA-phosphohydrolase. A considerable amount of DG thus formed was acylated to TG by the supernatant fraction while that in the pellet fraction was incorporated into TG and polar lipids.

The lipase activity of the supernatant fraction was found to be quite high. Most of these lipases were precipitated when the ammonium sulfate concentration was raised from 40-60% saturation.

In an attempt to test the ability of membrane-bound PA-phosphohydrolase to hydrolyze soluble substrate, phosphatidic acids (^{14}C-oleate) synthesized by Cell-Free Extract of M. smegmatis were extracted, purified and resuspended in 5% BSA. The "soluble" PA thus obtained was added to a cell-free system containing 4 mg washed 30,000 xg/1 hr. pellet and other co-factors. Effects of CTP, EDTA, and triose phosphates on its hydrolysis were also studied. Results are presented in Table 32.

It is evident that soluble phosphatidic acids are being hydrolyzed by the membrane-bound enzyme. However, unlike the mammalian system, a preference for soluble substrate by the membrane-bound PA-phosphohydrolase was not observed.

Table 31

A Study of Phosphatidic Phosphohydrolase Activity in the Pellet and Supernatant Fractions and in the Various Ammonium Sulfate Fractionated Supernatant Fractions Prepared From M. Smegmatis Cell-Free Extracts

Each Tube Contains 200 µl "pre-synthesized Membrane Lipids"	Distribution of ^{14}C-glycerol (DPM)				
	Water Soluble (after C:M Extract)	Triglyceride	Diglyceride	Phosphatidic Acids	Other Polar Lipids
Control (0 time)	4,650	590	1,570	65,200	5,090
+ Tris buffer .05M, pH 7.5	6,390	680	3,270	55,180	4,940
+ Pellet	7,610	1,980	5,740	49,260	6,170
+ Supernatant	12,480	9,600	4,330	43,300	3,760
+ 0-20% AMS	10,290	1,000	3,580	53,870	3,090
+ 20-40% AMS	13,000	970	4,010	46,860	3,460
+ 40-60% AMS	38,540	630	1,150	25,220	1,870

Conditions: covered in text

Table 32

Activity of Phosphatidic Acid Phosphohydrolase in the Washed Pellet Fractions Prepared From M. Smegmatis Cell-Free Extracts Against "Soluble" Phosphatidic Acids*

	Distribution of ^{14}C-oleate (cpm)			
	Triglyceride	Diglyceride	Fatty Acid	Polar Lipids
Control	38	116	70	2,186
+ 2.5 µm 3PGA	38	120	68	2,946
+ 2.5 µm 2,3-DPGA	46	122	76	4,614
+ 5 µm EDTA + 10 µm Mg^{2+}	124	140	64	1,178
+ 10 um EDTA + 15 µm Mg^{2+}	148	200	70	5,814
+ 0.5 µm CTP	44	132	58	818
+ 2 µm CTP	40	118	66	8,994

Conditions: 4 mg washed 30,000 xg/1 hr. pellet, 100 µmoles phosphate buffer, 0.1 µmoles CoA, 5 µmoles ATP, 5 µmoles Mg^{2+}, 5 µmoles NaF and 5 µmoles mercaptoethanol in a final volume of 1 ml.1 hr. incubation at 35C

* phosphatidic acids (^{14}C-oleate) synthesized by cell-free extract of M. Smegmatis were extracted, purified and dissolved in a small amount of ethyl ether, suspended in 5% BSA. The ET_2O was evaporated slowly and the PA was suspended in BSA by mechanically shaking the tube vigorously
PA (^{14}C-oleate) Ca 25,550 dpm added

No conclusive evidence concerning the effects of CTP, EDTA, and triose phosphate on the enzyme was obtained.

4. Estimation of Molecular Weights of Lipid Synthesizing Enzymes. The molecular weights of the various lipid synthesizing enzymes were estimated by ultrafiltration. Supernatant proteins were filtered through ultrafilters of varying pore size. The procedures used are covered in Materials and Methods.

Various filtrates containing proteins of different molecular weights were tested against the "pre-synthesized membrane-bound lipids". The results are presented in Table 33.

The formation of much greater amounts of labeled lipids in experiments where total supernatant or filtrate after xm100A filtration was used in comparison to control experiments may indicate the presence of ^{14}C-glycerol or ^{14}C-α-GP in the testing systems.

Upon examining the distribution of radioactivity in the lipids of the different tested systems, it is evident that the molecular weight of PA-phosphohydrolase is between 100,000 and 200,000 and that of the α-GP-acyltransferases less than 100,000.

From Table 33 it may be seen that the DG-acyltransferase activity was not present in the 100 to 300,000 fraction (through the XM-300 membrane but retained by the

Table 33

Ultrafiltration of Supernatant Fractions Prepared From M. Smegmatis Cell-Free Extracts: Enzyme Activity of Various Fractions of Filtrates

Each Tube contains 200 µl "Pre-synthesized Membrane-bound Lipids"	^{14}C-glycerol Incorporation (DPM)		
	Triglyceride	Diglyceride	Polar Lipids
0 Time Control	470	1,230	26,700
+ 200 µl Buffer (Tris 0.05M pH 7.5)	474	1,800	21,600
+ 200 µl Supernatant	6,020	2,080	30,970
+ 200 µl xm300-xm100A filtrate*	570	2,060	19,200
+ 200 µl xm100A filtrate	530	1,960	38,330

Conditions: procedure for ultrafiltration is covered in Materials and Methods
25 µmoles Mg^{2+} was added to each tube, 30 minutes incubation at 35C.

* xm300-xm100A filtrate: filtrate passed through the xm-300 membrane but retained by the xm100A membrane

xm100A filtrate: filtrate passed through the xm100A membrane

XM-100A membrane), and it was not present in the fraction which passed through the XM-100 membrane. It would appear from this data that this enzyme either has a molecular weight over 300,000 or it was inactivated in the filtration process.

VII. Acylation of Diglycerides to Triglycerides.

In vivo as well as in vitro evidence indicating diglycerides act as a direct precursor for triglyceride synthesis in mammalian tissues has been presented by various investigators. In an attempt to determine whether DG could be acylated to TG in Cell-Free Extracts of M. smegmatis or whether certain phosphatides act as intermediates in TG formation, various concentrations of diolein, phosphatidic acid, and cardiolipin were added to a cell free system in which the incorporation of ^{14}C-oleate into lipids has been demonstrated.

Results presented in Table 34 tentatively indicate the direct precursor role of DG in TG synthesis; upon the addition of 1 or 3 μmoles of diolein there was 8.4 and 18.2 μmoles, respectively, of ^{14}C-oleate incorporated into TG above that of the control tube.

To obtain further evidence to substantiate these experiments ^{14}C-glycerol-1,2-diolein and ^{14}C-glycerol-1,3-diolein were incubated with ammonium sulfate salt

Table 34

'Effects of Added Diglycerides and Phosphatides on the Incorporation of ^{14}C-oleate into Lipids by Total Cell-Free Extracts of M. Smegmatis

	^{14}C-oleate Incorporation (nmoles)		
	TG	DG	Origin
Control	13.0	0.51	3.80
+ 1 µmole DO	21.4	4.06	2.56
+ 1 µmole PA	9.1	0.39	2.03
+ 1 µmole DO + 1 µmole PA	7.39	0.46	1.50
+ 3 µmoles DO	31.2	3.01	1.97
+ 3 µmoles PA	6.58	0.26	1.36
+ 3 µmoles DO + 3 µmoles PA	1.86	0.20	0.70
+ 0.1 µmoles Cardiol*	14.9	0.55	2.52
+ 1 µmoles "	12.0	0.79	3.62
+ 3 µmoles "	4.34	0.37	2.57
+ 6 µmoles "	2.05	0.36	0.77

Conditions: 14 mg total cell-free extract proteins, 50 nmoles ^{14}C-oleate (sp. act. 5 µCi/µmoles) ATP 10 µmoles, CoA 0.25 µmoles, α-GP 7 umoles, Mg^{2+} 10 µmoles, Ni^{2+} 10 µmoles in a final volume of 1.5 ml incubated at 29C for 1 hr.

* Cardiol = cardiolipin

precipitated supernatant proteins and other necessary co-factors for lipid biosynthesis (Table 35).

The low counts in TG when 1,3-diolein was included in the cell free system indicated that this compound was not a suitable substrate for the esterification reactions. The fact that the addition of 5 μmoles of ATP in the incubation system did not increase radioactivity in any of the lipids demonstrates that the amount of DG being hydrolyzed to glycerol and then used as substrate for de novo lipid synthesis was negligible.

When ^{14}C-glycerol-1,2-diolein was used as a substrate it was esterfied to form TG by both the 0-20% and 20-40% AMS precipitated proteins, although a considerably greater amount of TG was found in the latter fraction. Such a finding is in agreement with results observed earlier (Table 29).

The incorporation of 1,2-diolein in TG by 20-40% saturation AMS precipitated supernatant proteins of M. smegmatis Cell-Free Extract was followed over a time period of 75 minutes (Figure 14). The results indicate that there was a gradual decrease of radioactivity in the DG fraction up to 45 minutes, after which the amount of label in DG remained constant. Although there was a gradual increase in the incorporation of labeled substrate in the TG, its increase does not correspond to the greater disappearance

Table 35

Incorporation of ^{14}C-glycerol-diolein in Triglyceride by Ammonium Sulfate Salt Precipitated Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts

			DPM Per Mg of Protein		
			Triglyceride	Monoglyceride	Polar Lipids
^{14}C-glycerol 1,2-Diolein	+ 5 umoles ATP	0-20% AMS	275	59	83
		20-40% AMS	722	68	136
	NO ATP	0-20% AMS	135	61	40
		20-40% AMS	461	78	42
^{14}C-glycerol 1,3-Diolein	NO ATP	20-40% AMS	46	23	33
	+ 5 umoles ATP	20-40% AMS	36	30	20

Conditions: 3-4 mg ammonium sulfate precipitated proteins, ^{14}C-glycerol-1,2-DO (632 nmoles) 54,154 dpm or ^{14}C-glycerol-1,3-DO (600 nmoles) 60,967 dpm, 5 µmoles, oleoylCoA 60 nmoles, 3 mg BSA in a final volume of 1 ml. 1/2 hr. incubation at 35C.

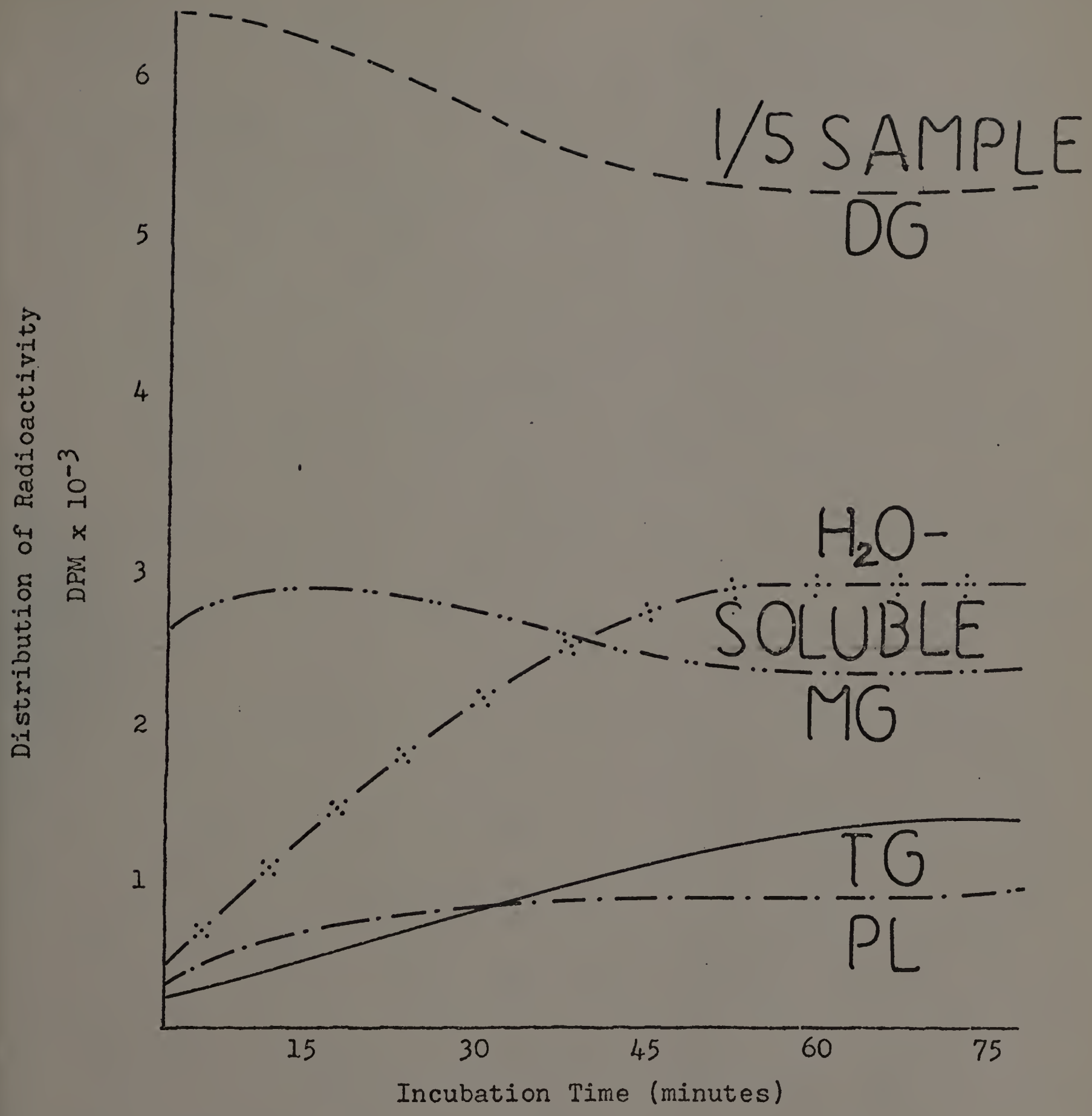

Figure 14

A Study of Esterification of DG by 20-40% AMS Precipitated Supernatant Proteins Prepared From M. Smegmatis Cell-Free Extracts.

Conditions same as for Table 35 with varying incubation time. 5 umoles ATP included.

of DG. A closer examination of the curves reveals that a considerable amount of the added DG was being hydrolyzed into water soluble material, probably glycerol. The nearly parallel relationship between the curves of DG and MG may indicate that the MG was an intermediate in the hydrolytic process.

The slight increase in radioactivity in phosphatides may be a result of the acylation of the ^{14}C-α-GP formed from ^{14}C-glycerol and ATP by the action of glycerol-kinase.

DISCUSSION

The dynamic role of lipids in bacterial metabolism is evident by the rapid and profound qualitative and quantitative changes which occur in these compounds upon alteration of environmental conditions. Many studies have shown that alterations in pH, medium composition, oxygen tension and cell age rapidly and radically change the cells lipid components.

The mycobacteria, partly because of their high lipid content and partly because of their role in disease production have been selected by biochemists as model organisms to study bacterial lipid composition and metabolism. Until recently, however, study of the neutral lipid components of these organisms has been neglected. This study was initiated in order to fill this gap. It has been directed at discerning the major pathways extant in triglyceride biosynthesis and has been concerned, in addition, to determining the nature of the regulatory mechanisms involved in this pathway.

In mammalian tissues three pathways have been delineated as being involved in TG biosynthesis: 1. the α-GP pathway (81); 2. the MG pathway (24); and 3. the DHAP pathway (47). Previous work from this laboratory (11) has

shown that the MG pathway does not exist in M. smegmatis. The relative importance of the other two pathways in TG biosynthesis is not known.

1. α-GP as the Primary Precursor for TG Synthesis.

The stereospecific distribution of fatty acids in TG's isolated from mycobacteria has been described (157). The substrate specificity of α-GP-acyltransferases in animal systems is quite controversial (12, 41, 54, 94, 97, 98, 114, 117 and 4, 5, 6, 66, 88, 93, 96, 110, 122, 142, 153, 164). On the other hand, greater selectivity of fatty acids in the acylation of DHAP than in the acylation of α-GP has been demonstrated (46). These facts together with Goldman's finding that the initial step of glycerol metabolism in M. tuberculosis $H37R_A$ is oxidation followed by phosphoxylation to DHAP (43) and the results of this study showing that the incorporation of ^{14}C-oleate into lipids is stimulated by the addition of dihydroxyacetone phosphates (Table 11) may lead one to assume that in M. smegmatis TG might be synthesized via the DHAP pathway. Experiments performed in this study with labeled glucose, fructose 1,6-diphosphate and 3-phosphoglycerate (Tables 14 to 17) have shown, however, that these compounds and in particular the glycolytic intermediate DHAP were not acylated to any extent.

It was shown indirectly on the other hand, that α-GP was the probable primary acyl acceptor (Tables 2, 3, 4, 5,

7 and 8). These experiments indicated that inclusion of α-GP in the incubation mixture caused a shift in incorporation of labeled fatty acid from TG to polar lipids (particularly PA). This effect was more pronounced in cell free preparations from glucose grown cells than from glycerol grown cells. Since the total incorporation of labeled fatty acid was generally not increased when α-GP was added, it appears that the observed increase resulted from a competition for the added radioactive substrate between α-GP and an unidentified TG precursor. Such an unidentified endogenous precursor has been reported in yeast by Johnston and Paltauf (71).

Direct evidence was presented which indicated that α-glycerophosphate acted as a primary acceptor for TG synthesis in M. smegmatis was obtained in studies where ^{14}C-α-GP or ^{14}C-glycerol was used.

It has been found that ^{14}C-α-GP and ^{14}C-glycerol were successfully incorporated into PA, polar lipids, DG, and TG by pellet fraction, supernatant fraction, and 0-20% and 20-40% AMS precipitated supernatant proteins of M. smegmatis Cell-Free Extract (Tables 18 and 26).

Results obtained from experiments where the incorporation of ^{14}C-α-GP into various lipids vs. time was studied (Figures 4 and 5) tend to indicate that the ^{14}C-α-GP was first acylated to a polar lipid which in turn served as an intermediate in the formation of DG and TG. Evidence

indicating that such a polar lipid was phosphatidic acid was also presented (Tables 29, 30 and 31).

It may also be seen that the α-GP-acyltransferase enzymes were found in both the pellet and supernatant fractions of M. smegmatis Cell-Free Extracts (Tables 8 and 26). These enzymes were precipitated from the supernatant protein fraction by 20-40% saturation ammonium sulfate (Table 26).

Low concentrations (to 3mM) of ATP and CTP were found to stimulate the α-GP-acyltransferase enzymes (Figures 7, 8, and 9, 10). Slight enhancement of their activities by low concentration of Mg^{2+} was also observed. However, high concentration of this ion (above 10 mM) was found to inhibit the enzymes (Table 21). Inhibition of the activity of this group of enzymes was also observed upon addition of fluoride ion (Tables 24 and 25), Tween-20 (Table 29) and upon freezing and thawing (Table 28).

Upon ultrafiltration of the supernatant fraction, the α-GP-acyltransferase activity was detected in the fraction of less than 100,000 molecular weight (Table 33).

2. Phosphatidate Phosphohydrolase. Phosphatidate phosphohydrolase is an important enzyme in the regulation of neutral lipid and phospholipid synthesis. In mammalian systems there appears to be two separate enzymes; the cytosol and the membrane-bound PA-phosphohydrolase. This

enzyme's activity was detected in both the supernatant and pellet fractions of M. smegmatis Cell-Free Extract. It appears, however, that the enzyme is a single entity. This conclusion is based on the observations that the activity in both the supernatant and pellet fractions are Mg^{2+} -dependent (Tables 19 and 20), and that in contrast to the mammalian systems, there was no preference for soluble substrates by the membrane-bound enzyme (Tables 31 and 32). Furthermore, the similarity of response of this enzyme from both the supernatant and pellet fractions to various activators and inhibitors tends to indicate the presence of one hydrolase.

The Mg^{2+} -dependency of the phosphatidate phosphohydrolase enzyme was shown (Tables 19 and 20). A gradual increase in activity was observed when the concentration of Mg^{2+} in the incubation mixture was gradually increased up to 5 mM (Table 21 and Figure 7), however, concentration above 5 mM were found to be inhibitory.

Experiments in which the incorporation of ^{14}C-oleate (Tables 10 and 11) and ^{14}C-α-GP (Table 24 and Figure 12) by lipids were studied revealed that the uptake of the labeled substrates by neutral lipids was greatly stimulated by the inclusion of Na^+ or K^+ ions in the incubation system. The increase of neutral lipid formation was paralleled by a corresponding decrease in the label in the polar

lipid fraction. Since over 90% of the polar lipids was phosphatidic acid (Tables 29, 30 and 31), it appeared that the effect of the monovalent cations was the activation of the phosphatidate phosphohydrolase.

Evidence has been presented which indicated that the activity of this enzyme was inhibited by Tween-20 (Table 30). Its activity was not affected by low concentrations of fluoride ion (Tables 23, 25 and 30), although high concentrations of this ion (50 mM) was found to be inhibitory.

Based on the results obtained from ultrafiltration experiments where phosphatidate phosphohydrolase activity was detected in the 100 to 300,000 molecular weight fraction, it appeared that the molecular weight of this enzyme was between 100,000 and 300,000 (Table 33).

Attempts to study the effect of ATP and CTP on the activity of this enzyme have been made. It was evident that these nucleotides act both as positive and negative modulators of lipid biosynthesis according to their intracellular concentrations (Figures 7, 8, 9 and 10). Since the acylation of α-GP and the formation of DG and TG are simultaneously increased and/or decreased, the mode of action of these nucleotides can not be determined. Smith and Hubscher (139) and Johnston et. al. (72) have suggested that ATP (and indirectly CTP) may assist in catalyzing the formation of acyl-CoA's from endogenous fatty acids which

would be preferentially utilized by the acyltransferases in esterifying α-GP. The observations in this study that small amounts of CTP stimulated the incorporation of labeled oleate by lipids (Table 9) tends to suggest that in M. smegmatis, at least, such a proposed mode of action of this nucleotide may not be of importance.

In order to ascertain the true effect of ATP and CTP on phosphatidate phosphohydrolase, it would be necessary to purify the enzyme and study the effect of the nucleotides on the purified enzyme. The observation that the activities of the α-GP-acyltransferases and the PA-phosphohydrolase could be separated by ultrafiltration (Table 33) and that repeated freezing and thawing inhibited the activity of DG-acyltransferase (Table 28) but not that of the PA-phosphohydrolase presents a possible approach to at least a partial purification of the PA-phosphohydrolase enzyme.

3. DG-Acyltransferase. Indirect evidence was presented in Table 34 that diglycerides serve as direct precursors of TGs. The addition of diglycerides to cell free incubation mixtures resulted in increased incorporation of label by the TG fraction. Other experiments (Table 35 and Figure 14) using glycerol labeled 1,2-DG further substantiated the precursor relationship of diglycerides. In these experiments the labeled DG was acylated to TG by various ammonium sulfate precipitated fractions of cell free extract.

It was evident that the enzyme DG-acyltransferase was present in both the supernatant fraction (Tables 2 and 3) and the pellet fraction (Table 8) of M. smegmatis Cell-Free Extracts. This enzyme's activity was found to be much greater in the supernatant fraction (Figures 4 and 5). By making the supernatant fraction to 40% saturation with ammonium sulfate, the DG-acyltransferase was rendered sedimentable with nucleic acids and lipoprotein; when the crude supernatant fraction was used, most of the enzyme was precipitated by 0-20% AMS (Tables 26 and 28). However, when the nucleoproteins in the supernatant were removed with $MnSO_4$ prior to AMS fractionation, higher activity of this enzyme was found in the 20-40% AMS fraction (Tables 29 and 35).

Evidence was presented that Mg^{2+} stimulated DG-acyltransferase activity (Tables 19 to 21). In addition, it was also shown that ATP, when added in low concentrations, appeared also to stimulate this enzyme (Figures 7 and 8). Experiments outlined in Table 35 showed that when ATP was added to a concentration of 5 mM, a two fold increase in TG formation from ^{14}C-glycerol-1,2-diolein occurred. Similar results were found when CTP was substituted for ATP in the reaction mixture (Figures 9 and 10).

Data presented in Tables 10 and 11 have shown that Na^+ and K^+ caused an increase in incorporation of labeled fatty

acid in TG. Whether the stimulation of incorporation was due to action of these ions on the DG-acyltransferase or on the PA-phosphohydrolase enzyme is not known. Figure 12, however, does clearly show that small amounts of Na^+ do cause a greater increase in labeling of TG than DG and one would suspect the DG-acyltransferase as well as the PA-phosphohydrolase enzyme was stimulated.

4. Effects of Triose Phosphates. With the exception of α-glycerophosphate, all of the triose phosphates tested enhanced incorporation of ^{14}C-fatty acid by lipids, particularly in the case of the triglycerides (Tables 4, 5 and 6). When the magnitude of the stimulatory effect of various glycolytic products was compared (Table 11), it was found that the degree of stimulation of incorporation by glyceraldehyde 3-phosphate and di-hydroxyacetone phosphate was the same. However, a greater enhancement was observed with 2,3-diphosphoglycerate. These results tend to suggest that the mode of action of the glycolytic products is in an indirect fashion, by generating ATP rather than by some direct action upon the lipid synthesized enzymes. The similarity in the pattern of activation and/or inhibition of lipid synthesis between the different concentrations of 2,3-diphosphoglycerate (Tables 8 and 11), and those of ATP (Figure 8) supported this view.

From the foregoing discussion it is evident that the major route operable in M. smegmatis for the biosynthesis

of TG is the α-GP pathway. This pathway is the major one which operates in animal systems. The major point of dissimilarity between the bacterial and animal systems is in the nature of the PA-phosphohydrolase enzyme. This enzyme appears to be a single entity in the bacterial system while it is two separate enzymes in the animal system, being totally Mg^{2+} -dependent, stimulated by Na^{+}, K^{+} and inhibited by Tween-20 and F^{-}.

Johnston and Paltauf (71) have reported an unidentified endogenous TG precursor in yeast. Evidence has been presented in this study that a similar acceptor is present in cell free extracts of M. smegmatis. Conclusions drawn from the various experiments performed suggest that this compound is 1,2-DG.

In animal tissues, the regulation of TG-formation by hormones is undoubtedly of overriding importance. It seems logical to speculate that certain hydrophilic small molecules are responsible for regulating TG synthesis in microorganisms. It has been found that small amounts of ATP or CTP stimulate the activities of α-GP-acyltransferase and that of DG-acyltransferase. However, no conclusive results have been obtained regarding the effect of these nucleotides on the key enzyme PA-phospholydrolase. The true mechanisms of modulation of ATP, CTP, and other possible effectors on this enzyme can not be determined until the

enzyme is separated from the other lipid synthesizing components and studied separately. A possible partial purification process has been suggested.

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